Centro de Investigación en Alimentación y Desarrollo, A.C.

POTENCIAL EFECTO CARDIOPROTECTOR DE LECHES FERMENTADAS CON *Lactococcus lactis* ASOCIADO A LA REGULACIÓN DE LOS NIVELES DE LÍPIDOS Y LA MICROBIOTA INTESTINAL EN MODELOS *In vivo*

Por:

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RESUMEN

El objetivo del presente estudio fue evaluar el efecto cardioprotector de leches fermentadas (LF) con cepas específicas de *Lactococcus lactis* NRRL B-50571 (LF-571), NRRL B-50572 (LF-572) y NRRL B-50600 (LF-600) mediante la regulación de los niveles de lípidos, y evaluar la participación de la microbiota intestinal y fracciones peptídicas. El primer objetivo particular consistió en evaluar el efecto hipocolesterolémico de las LF en modelo murino. Los resultados mostraron que el consumo de LF-572 y LF-600 reducen significativamente los niveles de colesterol total y colesterol no-HDL (C-LDL+C-VLDL) plasma en un 20 y 23 %, respectivamente ($p<0.05$). Además, las LF previnieron la acumulación de lípidos hepáticos. El segundo objetivo consistió en investigar la asociación de la microbiota intestinal y el perfil de lípidos por el consumo de las LF. El consumo de LF-572 aumentó la abundancia de *Ruminococcaceae*, *Lactobacillaceae*, *Oscillospiraceae* y *Lachnospiraceae* en heces. Estas familias se asociaron negativamente con el colesterol total y no-HDL ($p<0.05$). Además, el consumo de LF aumentó la abundancia de ácidos grasos de cadena corta (AGCC). De los AGCC, solo butirato se asoció negativamente con el colesterol total y no-HDL, y positivamente con C-HDL ($p<0.05$). Por lo tanto, la microbiota intestinal podría estar relacionada con el efecto hipocolesterolémico de las LF. En el tercer objetivo, se investigó el potencial efecto hipocolesterolémico de fracciones peptídicas provenientes de LF-572 y de LF-600 mediante ensayos *in vitro*. Los resultados mostraron que las LF liberan fracciones peptídicas potencialmente bioaccesibles con capacidad para inhibir la formación de micelas de colesterol e inhibir la actividad de HMG-CoA reductasa. Lo anterior, sugiere que la regulación de los niveles de colesterol podría estar dado en parte por péptidos capaces de inhibir la absorción y síntesis de colesterol. En el último objetivo se evaluó el efecto hipocolesterolémico por la administración de fracciones peptídicas (<10 kDa) de LF-572 y LF-600 en ratas Sprague-Dawley hipercolesterolémicas. Los resultados mostraron efecto hipocolesterolémico por ambas fracciones. En específico, la fracción de LF-572 redujo significativamente los niveles de colesterol total, colesterol no-HDL, triglicéridos, ácidos grasos libres y el índice aterogénico en plasma. Además, se observó una mayor excreción fecal de colesterol. Finalmente, la caracterización parcial de los péptidos de LF-572 reveló las posibles secuencias peptídicas asociadas al efecto hipocolesterolémico de LF-572. Por todo lo anterior, las LF, específicamente LF-572 presenta

potencial efecto cardioprotector debido a su capacidad para reducir los niveles de colesterol, la cual pudiera ser utilizada como coadyuvante en el manejo de la hipercolesterolemia.

Palabras clave. Hipercolesterolemia**,** bacterias acido lácticas, alimentos funcionales, efecto hipocolesterolémico.

ABSTRACT

The aim of this study was to evaluate the cardiprotective effect of fermented milks (FMs) with *Lactococcus* (*L.*) *lactis* NRRL B-50571 (FM-571), NRRL B-50572 (FM-572) and NRRL B-50600 (FM-600) by the regulation of lipid levels and evaluate the participation of of gut microbiota and bioactive peptides. Firstly, the hypocholesterolemic effect of FM-571, FM-572 and FM-600 were evaluated in rats. The results showed that the consumption of these FMs, specifically FM-572 and FM-600 reduced total cholesterol and non-HDL (LDL-C+VLDL-C) in plasma by 20 and 23%, respectively. Moreover, less hepatic lipid accumulation was observed by the effect of FMs. Furthermore, the effect of fermented milks on gut microbiota was evaluated. From all fermented milks, the consumption of FM-572 showed effect in the increase of *Ruminococcaceae, Lactobacillaceae, Oscillospiraceae* and *Lachnospiraceae.* These families were negatively associated with total cholesterol and non-HDL. Also, the consumption of FMs enhanced the levels of SCFAs. From all SCFAs, only butyrate was negatively correlated with total and non-HDL cholesterol, and positively correlated with HDL-C ($p<0.05$). Hence, gut microbiota might be involved in the hypocholesterolemic effect of these FMs. Next, the potential hypocholesterolemic effect of peptide fractions derived from FM-572 and FM-600. Results indicated that these FMs released bioaccessible peptide fractions with the capacity to decrease micellar solubility of cholesterol and inhibit HMG-CoA reductase. Thus, results suggest that the hypocholesterolemic effect may involve peptides capable of inhibiting cholesterol absorption and cholesterol synthesis. Furthermore, the effect on the regulation of lipid levels by the administration of peptide fractions from FM-572 and FM-600 to hypercholesterolemic rats was evaluated. The findings indicated that these fractions exert hypolipidemic effect. Specifically, the administration of peptide fractions from FM-572 decreased total cholesterol, non-HDL, triglycerides, and non-esterified fatty acids in plasma. Also, a high fecal excretion of cholesterol was observed. Finally, partial characterization of peptides revealed specific peptides sequences involved in the hypocholesterolemic effect of FM-572. Hence these findings suggest that peptide fractions derived from fermentation exert hypocholesterolemic effect. Collectively, our findings demonstrated that the consumption of fermented milks, specifically, FM-572 present cardiprotective effect and this FM can be used as a coadjutant in the management of hypercholesterolemia, particularly for reducing the cardiovascular risk.

Key words: Hypercholesterolemia, lactic acid bacteria, functional foods, hypocholesterolemic effect.

1. SINOPSIS

1.1. Justificación

De acuerdo con cifras de estudios epidemiológicos, desde hace varias décadas, las enfermedades cardiovasculares (ECV) siguen en aumento. (Khan et al., 2020, Mozaffarian *et al*., 2016). Las EVC engloban enfermedades relacionadas al sistema circulatorio y al corazón, como, por ejemplo, la enfermedad cerebrovascular y la cardiopatía isquémica (Khan *et al*., 2020, Mozaffarian et al., 2016). Un estudio demostró que los factores de riesgo más significativos, atribuibles al desarrollo de las ECV fueron, los factores dietéticos, hipertensión, hipercolesterolemia y sobrepeso-obesidad (Virani et al., 2020). Se ha descrito que estos factores de riesgo son potencialmente reversibles lo que proporciona una gran oportunidad para su abordaje (Timmis *et al*., 2022). Además, estudios recientes han evidenciado que la alteración de la microbiota intestinal juega un papel muy importante en el desarrollo de las ECV, a través de su implicación en los factores de riesgo antes mencionados (Witkowski *et al*., 2020).

En este sentido, la dislipidemia, en específico, la reducción de hipercolesterolemia, con la reducción de colesterol de las lipoproteínas de baja densidad (C-LDL), ha sido el objetivo de múltiples enfoques terapéuticos. De acuerdo con las guías clínicas internacionales para el manejo de lípidos, la reducción de C-LDL, requiere un enfoque múltiple que incluya inicialmente, la mejora en el estilo de vida (*v.g*. actividad física, alimentación) y, si es requerido, complementar con un tratamiento farmacológico (Aygun y Tokgozoglu, 2022). El consumo de alimentos saludables, así como la adopción de patrones dietéticos debe integrarse como parte del mejoramiento al estilo de vida (Agarwala *et al*., 2022). A este respecto, el consumo de alimentos funcionales ha sido recomendado por sus efectos benéficos demostrados en la salud cardiovascular. Particularmente, los productos lácteos fermentados funcionales muestran un beneficio potencial en la reducción de riesgo cardiovascular de acuerdo con estudios sistemáticos y de metaanálisis (Fontecha et al., 2019, Lovegrove y Hobbs, 2016). De hecho, este efecto, parece asociarse en la disminución significativa de C-LDL y de triglicéridos (Ziaei *et al*., 2021).

Se sabe que el efecto benéfico de los lácteos fermentados es cepa especifica, y que depende en gran medida en su capacidad para sobrevivir en el sistema gastrointestinal (probióticos), o bien para producir compuestos bioactivos durante el proceso fermentativo de la leche. En este sentido, se ha demostrado que el efecto hipocolesterolémico de las leches fermentadas se asocian a la presencia de probióticos (Ziaei *et al*., 2021). También, los lácteos fermentados son ricos en nutrientes, metabolitos y componentes bioactivos, los cuales son liberados durante el proceso fermentativo de la leche, como es el caso de los péptidos bioactivos. A pesar de que existe una amplia evidencia del papel benéfico de los péptidos bioactivos, son escasos los estudios que evidencian su potencial efecto en la regulación de los niveles de colesterol. El efecto hipocolesterolémico por péptidos derivados de proteínas lácteas ya ha sido reportado; sin embargo, la obtención de estos péptidos se ha realizado con el uso de enzimas específicas (*v.g*. pepsina, tripsina y neutrasa) (Nagaoka *et al*., 2001, Jiang *et al*., 2020, Lee y Youn, 2020). Se ha reconocido que las proteínas lácteas son fuente importante de péptidos con actividad biológica. Dichos péptidos se han obtenido mediante varias estrategias entre las cuales incluyen el empleo de enzimas específicas, mediante la digestión gastrointestinal y por la proteólisis bacteriana durante el proceso de fermentación de la leche. No obstante, varios estudios han demostrado que los péptidos obtenidos mediante proteólisis bacteriana han mostrado mejores propiedades en cuanto a eficacia y multifuncionalidad. (Raveschot et al.., 2018). En este sentido, cepas de *Lactococcus* (*L*.) *lactis* son caracterizadas por su alta capacidad proteolítica y fermentativa, por lo que el empleo de dichas bacterias es prometedor para obtener una diversidad de péptidos en la elaboración de leches fermentadas (Li *et al*., 2020).

En estudios previos se demostró que el consumo de leches fermentadas con cepas específicas de *L. lactis* (NRRL B-50572 y NRRL B-50600) mejoró los niveles de colesterol y triglicéridos en ratas espontáneamente hipertensas y sujetos pre-hipertensos (normocolesterolémicos) (Rodríguez-Figueroa et al., 2012, Beltrán-Barrientos et al., 2018). Sin embargo, es necesario consolidar estos hallazgos en modelos *in vivo* con hipercolesterolemia, con el fin de establecer el efecto benéfico cardiovascular asociado al mejoramiento del perfil de lípidos. Además, es necesario determinar si los péptidos generados durante la fermentación están asociados al efecto hipocolesterolémico. Es importante también resaltar que la microbiota intestinal podría jugar un papel importante en el metabolismo de lípidos, lo cual en una condición de disbiosis intestinal puede alterar el perfil de

lípidos, aumentando, por lo tanto, el riesgo cardiovascular. Por ello, resulta importante determinar si el consumo de las leches fermentadas puede mejorar la composición de la microbiota intestinal y ejercer un efecto benéfico a la salud cardiovascular.

1.2. Antecedentes

1.2.1. Hipercolesterolemia y su Papel en el Desarrollo de Enfermedades Cardiovasculares

La hipercolesterolemia es un desorden perteneciente a las dislipidemias y se caracteriza por niveles elevados de colesterol total (>240 mg/dL) y de C-LDL. Los niveles de C-LDL son elevados cuando se encuentran por arriba de 190 mg/dL cuando no se acompaña de otros factores de riesgo cardiovascular, o bien cuando se encuentran por arriba de 130 mg/dL con la existencia de otros factores de riesgo. El desarrollo de la hipercolesterolemia puede deberse a un estilo de vida inadecuado o bien por el factor genético, denominado, hipercolesterolemia familiar (Vrablik *et al*., 2020, McGowan *et al*., 2019). La hipercolesterolemia familiar proviene de una alteración genética asociadas a mutaciones en genes específicos que expresan proteínas claves en el metabolismo del colesterol. Principalmente, la frecuencia de las mutaciones se da en el receptor de C-LDL (LDLR), apolipoproteína B (ApoB) y de la Apoproteína Convertasa Subtilisina Kexina de tipo 9 (PCSK9). La afectación de la sobreproducción o bien, de la supresión de la expresión de estas proteínas causa alteración en el catabolismo de las C-LDL, aumentando los niveles en el torrente sanguíneo, con un riesgo prematuro de desarrollar aterosclerosis (Bouhairie y Goldberg, 2015).

1.2.2. Visión General del Metabolismo del Colesterol y Enfoques Terapéuticos para el Manejo de la Hipercolesterolemia

El colesterol es una molécula importante en el cuerpo humano ya que forma parte estructural de

las membranas celulares, es precursor de las sales biliares, de hormonas esteroideas y de vitamina D. Los niveles de colesterol en el cuerpo se encuentran regulados por un metabolismo complejo que incluye una regulación a nivel celular y molecular dictada por la acumulación (reserva hepática), absorción y excreción. En general, los niveles de colesterol en el cuerpo es el resultado del metabolismo exógeno y endógeno. En un estado de hipercolesterolemia, los enfoques terapéuticos pueden abordarse en cualquiera de las vías exógena y endógena para su regulación (Xu *et al*., 2020, Kapourchali *et al*., 2016). Además, en los últimos años se ha reconocido la importancia de la microbiota intestinal y su interacción con el metabolismo del colesterol (Xu *et al*., 2020, Vourakis *et al*., 2021).

1.2.2.1. Metabolismo Exógeno. La vía exógena implica esencialmente la absorción de colesterol dietario, lo cual inicia con la ingesta del colesterol (ésteres de colesterol) proveniente de los alimentos. En el lumen intestinal, los ésteres de colesterol son hidrolizados por la enzima colesterol esterasa o lipasa activada por sales biliares. Posteriormente, el colesterol libre, junto con otros lípidos son posteriormente emulsificados por las sales biliares, formando micelas mixtas. Los constituyentes lipídicos son luego absorbidos por las células epiteliales intestinales. En este punto, el transportador Niemman Pick C1-Like 1 (NPC1L1) facilita el transporte del colesterol intracelular. De hecho, Se ha reconocido el papel de NPC1L1 en la regulación del eflujo transepitelial del colesterol, donde también participan los transportadores de ATP-Binding Cassette 5 y 8 (ABCG5/8) (Lammernt y Wang, 2005). Este eflujo de colesterol en la dirección lumen-enterocito o enterocito-lumen define el grado de absorción de colesterol (Lammert et al., 2005, Luo et al., 2020).

En el lumen, una vez que los lípidos fueron absorbidos, las sales biliares son reabsorbidas de manera eficiente en el íleon distal, que posteriormente pasan nuevamente al hígado y a la vesícula biliar para ser nuevamente liberadas en el lumen intestinal en el proceso de emulsificación de lípidos, proceso al cual se le denomina circulación enterohepática de sales biliares (Cai y Chen, 2014). Con base en lo anterior, se han desarrollado fármacos hipocolesterolémicos. La ezetimiba, es un fármaco que bloquea NPC1L1, resultando en una menor absorción de colesterol (Nutescu *et al*., 2003). Por otra parte, la colestiramina, se une a las sales biliares, reduciendo la eficiencia de la

formación de micelas y, en paralelo, disminuyendo la reabsorción de las sales biliares. La disminución de la concentración de las sales biliares lleva a una regulación mediante retroalimentación negativa. La pérdida de las sales biliares se compensa por la síntesis *de novo* de sales biliares en la cual se expresa la enzima colesterol 7-α hidroxilasa (CYP7A1) (Stellaard y Lütjohann, 2014).

1.2.2.2. Metabolismo Endógeno. La distribución de colesterol en el organismo se da mediante diferentes procesos. No obstante, la participación de las diferentes lipoproteínas es un factor común en todos ellos. En primer lugar, los quilomicrones se ensamblan en el enterocito posterior a la absorción de lípidos dietarios. La función principal de los quilomicrones es la distribución de estos lípidos, principalmente triglicéridos, a tejidos específicos (*v.g*. tejido adiposo y muscular). Los triglicéridos son captados en estos tejidos en forma de ácidos grasos libres por la acción de la enzima lipoproteína lipasa (LPL) (Kersten, 2014). Posteriormente, los quilomicrones remanentes (pobres en triglicéridos) ricos en ésteres de colesterol son captados por el hígado para su catabolismo por el hígado (Xiao y Lewis, 2012).

La distribución de lípidos endógenos en el organismo, principalmente triglicéridos, se realiza mediante las lipoproteínas de muy baja densidad (VLDL), los cuales son ensamblada
s en el hígado. En general, los triglicéridos de las VLDL son captados por los tejidos (*v.g*. adiposo) por la enzima LPL. Debido a que LPL es insulinodependiente, se ha descrito que, en condiciones de resistencia a insulina, los niveles de VLDL incrementan resultando en hipertrigliceridemia (Sparks *et al*., 2012). La extracción de triglicéridos de las VLDL por LPL en los tejidos, conlleva a la formación de lipoproteínas de densidad intermedia (IDL). Los triglicéridos remanentes de IDL son luego transferidas a HDL3, mientras que el colesterol de las HDL3 se transfiere a las IDL. Esta trasferencia se realiza mediante la proteína de transferencia de ésteres de colesterol. Además, los triglicéridos también pueden ser captados por la lipasa hepática. El cambio de proporción de lípidos de las IDL da origen a las LDL, las cuales son ricas en ésteres de colesterol (Nakajima *et al*., 2011).

Las LDL derivan en su mayor parte de las VLDL y su función es el transporte de colesterol a los tejidos extrahepáticos. La distribución del colesterol en los diferentes tejidos requiere la

internalización o endocitosis de las LDL. Este proceso requiere el receptor LDLR, las LDL ricas en colesterol endocitadas son luego desensamblados por los lisosomas y los ésteres de colesterol son luego hidrolizadas por la enzima acil-CoA colesterol aciltransferasa (ACAT). El colesterol es utilizado por la célula, mientras que los receptores son reciclados (Duan *et al*., 2022). Las LDL no utilizadas son captadas en el hígado por LDLR. En la hipercolesterolemia familiar la mutación en el gen para LDLR conlleva a un aumento significativo de C-LDL en el torrente sanguíneo. Además, se ha descrito que la sobreexpresión de PCSK9 en la hipercolesterolemia familiar, lleva a una baja de los receptores de LDLR, ya que PCSK9 induce la degradación de dichos receptores durante el reciclaje. Por lo tanto, la baja densidad de LDLR lleva a un aumento significativo de C-LDL en la sangre. Las LDL son las lipoproteínas clínicamente más importantes debido su papel en la aterogénesis, por lo tanto, se han desarrollado estrategias prometedoras enfocadas en aumentar los receptores LDLR y en disminuir la actividad de PCSK9 (Lagace, 2014, Diffenderfer y Schaefer, 2014).

Las HDL son otra clase de lipoproteínas cuya función principal es el transporte reverso del colesterol, desde los tejidos periféricos al hígado. Las HDL son ensambladas en el hígado y en el intestino. El proceso de transferencia requiere de la actividad de la proteína *ATP-Binding Cassette A1* (ABCA1) presente en la superficie de los tejidos periféricos. El colesterol captado por las HDL es luego esterificado por la enzima lecitina colesterol acil transferasa (LCAT). Es importante destacar que las HDL presenta múltiples funciones, entre ellas, es la transferencia de proteínas (receptores) para el aclaramiento de lipoproteínas, la transferencia de lípidos con las IDL. Además, se han reportado efectos antiinflamatorios y antioxidantes (Duan *et al*., 2023, Rader, 2006, Soran *et al*., 2015). En este sentido, se han desarrollado estrategias terapéuticas para incrementar los niveles de HDL en la hipercolesterolemia, por ejemplo, en mejorar la actividad de LCAT (Freeman *et al*., 2020).

El metabolismo endógeno, involucra inicialmente la síntesis de colesterol, principalmente en el hígado y en el intestino. La síntesis inicia con la Acetil-CoA como sustrato, la cual está seguida de más de 20 pasos enzimáticos. Un paso enzimático importante en este proceso es la producción de ácido mevalónico a partir de la molécula 3-Hidroxi-3-metilglutaril CoA (HMG-CoA) catalizado por la enzima HMG-CoA reductasa. Esta enzima juega un papel clave que determina la velocidad

de síntesis de colesterol (Michos *et al*., 2019). Actualmente, la inhibición de la HMG-CoA reductasa por fármacos (*v.g.* estatinas) son utilizados para disminuir los niveles de C-LDL. La reducción de C-LDL se da por un agotamiento de colesterol intracelular, como consecuencia, los receptores LDLR son expresados para captar C-LDL del torrente sanguíneo, consiguiendo de esta forma disminuir el colesterol (Pang *et al*., 2020). Cabe resaltar que, el proceso de síntesis esta estrictamente regulado a nivel génico por el factor de transcripción SREBP2 (proteína de unión a elementos reguladores de esteroles). SREBP2 regula negativa o positivamente la expresión de HMG-CoA reductasa y los receptores LDLR (Duan *et al*., 2023). La regulación de los niveles de colesterol puede darse por diversos factores como lo es por la acción de compuestos bioactivos y metabolitos derivados de la microbiota intestinal.

1.2.3. Participación de la Microbiota Intestinal en el Metabolismo del Colesterol

El sistema gastrointestinal aloja una gran diversidad de microrganismos, principalmente bacterias, que coexisten con el hospedero. Se han estimado más de 100 trillones de bacterias dentro de dicho ecosistema. Funcionalmente, la microbiota participa activamente metabolizando compuestos, produciendo nutrientes y regulando procesos metabólicos e inmunes (Doré y Blottiere, 2015). Por lo tanto, el balance (eubosis) permiten un estado de salud adecuado. Por el contrario, una alteración en la composición y funcionalidad (disbiosis) potencialmente puede llevar al desarrollo de enfermedades como lo son las ECV (Jia *et al*., 2021). Por ejemplo, en la hipercolesterolemia se han descrito posibles variantes, principalmente en la alteración de la composición de los filotipos y la reducción de la diversidad (Rebolledo *et al*., 2017). Los estudios muestran la alteración de la abundancia de *Faecalibacterium, Veillonellaceae, Victivallaceae, Lactobacillaceae, Rumminococcaceae*, *Prevotella* y *Roseburia*, de los cuales varios de estos taxones mostraron asociación significativa positiva o negativa con los niveles de C-LDL, C-HDL o triglicéridos. (Morales *et al*., 2022, Guo *et al*., 2022, Yun *et al*., 2020). Se ha descrito que los cambios en el perfil de lípidos asociados a la microbiota se relaciona especialmente por un desbalance de los metabolitos producidos, principalmente, ácidos grasos de cadena corta (AGCC), sales biliares secundarias, óxido de trimetilamina (TMAO) y lipopolisacárido (LPS**).** En la **Figura 1** se ejemplifican las vías de dichos compuestos en el metabolismo del colesterol.

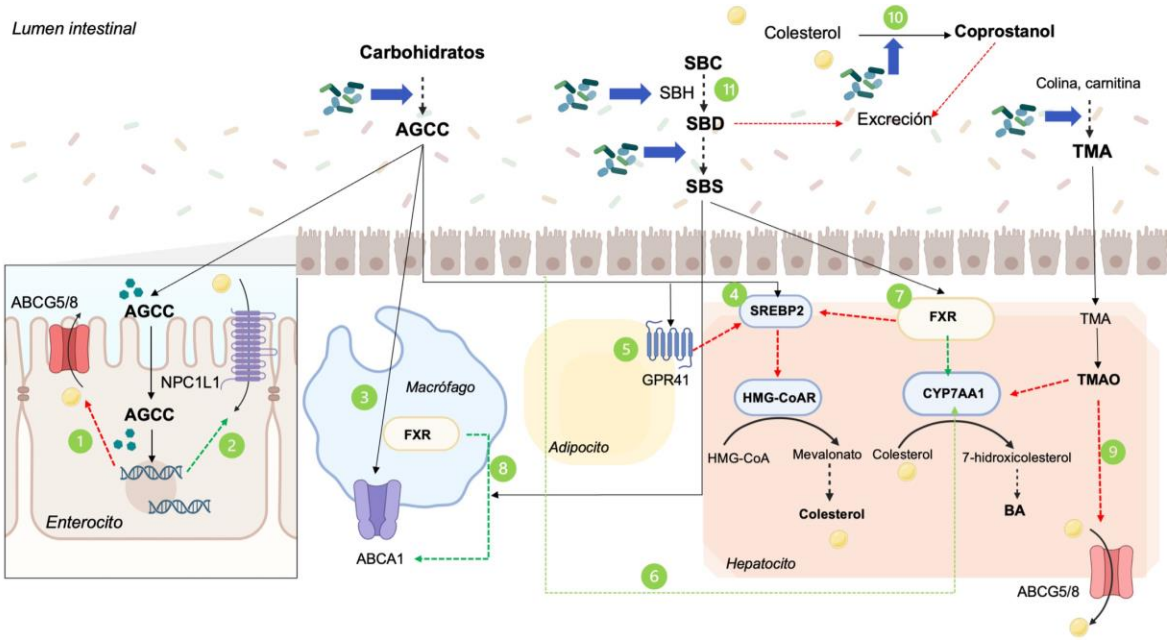

Figura 1. Participación de la microbiota intestinal y sus metabolitos en el metabolismo de colesterol. Efecto los AGCC sobre 1) Activación de ABCG5/8; 2) Supresión de NPC1L1; 3) Activación de ABCA1; 4) Modulación de SREPB2; 5) Activación de GPR41-léptina; 6) Activación de CYP7A1; 7) Activación de FXR hepático; 8) Activación de FXR en macrofágo; 9) Supresión de ABCG5/8; 10) Producción de coprostanol; 11) Efecto en la reducción micelar por desconjugación de sales biliares. (Fuente: elaboración propia en BioRender.com).

Los AGCC son los principales metabolitos producidos por la microbiota intestinal, que incluyen principalmente, acetato, propionato y butirato. Estos metabolitos resultan principalmente del metabolismo de carbohidratos no digeridos. No obstante, también pueden generarse por el metabolismo de aminoácidos (Chambers *et al*., 2018, Morrison *et al*., 2016). Se ha descrito el papel de los AGCC en el metabolismo del colesterol exógeno y endógeno. A nivel exógeno pueden modular el eflujo transepitelial del colesterol mediante la expresión o supresión de NPC1L1 y ABCG5/8 en los enterocitos. A nivel hepático, pueden inhibir directamente la síntesis de colesterol a través de la HMG-CoA reductasa o por la supresión de SREBP2. La inhibición de la síntesis puede ocurrir mediante la activación del receptor acoplado a la proteína G41 (GPR41) en el tejido adiposo. Lo anterior lleva a la producción de léptina, lo cual consigue suprimir SREBP2. La expresión de CYP7A1 y la consiguiente síntesis de sales biliares permite la regulación de colesterol por parte de los AGCC (Alvaro *et al*., 2008, Hara *et al*., 1999, Jia *et al*., 2021).

Las sales biliares secundarias son otro de los compuestos que tienen una implicación significativa

en el metabolismo de lípidos y de las sales biliares mismas. Se obtienen tras varios procesos enzimáticos (desconjugación, deshidroxilación, deshidrogenación y epimerización). El efecto regulador de estos compuestos se da por múltiples mecanismos, a nivel exógeno las sales biliares desconjugadas por los microorganismos con la presencia de la enzima hidrolasa de sales biliares (HSB) pueden inhibir la formación de micelas de colesterol (Gérard, 2013). Las sales biliares secundarias, por otra parte, pueden activar el receptor X farnesoide (FXR) en el enterocito, hepatocito o macrófago. Como consecuencia, la activación de FXR puede modular el eflujo de colesterol por la activación de ABCG5/8. Además, la activación de ABCA1 en los macrófagos promueve la salida de colesterol mediante el transporte reverso del colesterol mediada por las HDL.

La supresión de la síntesis de colesterol es otro de los efectos de las sales biliares secundarias. Se han descrito una variedad de estructuras químicas de estas sales biliares, la modulación del metabolismo del colesterol está dada por la especificidad para la activación de las diferentes rutas. Por ejemplo, el ácido quenodexicólico, ácido deoxicólico y el ácido litocólico presentan más afinidad para activar FXR, lo cual está determinado por la abundancia de taxones específicos para metabolizarlos (Sato *et al*., 2021, Gérard, 2013). Otras de las moléculas microbianas potencialmente aterogénicas es el TMAO. Inicialmente, la L-carnitina y colina son precursores de TMA, que posteriormente se oxida en el hígado, produciendo TMAO. Sus propiedades aterogénicas se deben a que el TMAO puede inhibir el transporte reverso del colesterol. Por otra parte, esta molécula puede inhibir la expresión de CYP7A1 disminuyendo, por la tanto, la conversión de colesterol a sales biliares. No obstante, los estudios recientes sobre la influencia clínica del TMAO en el desarrollo de la aterosclerosis no está clara (Zhu *et al*., 2020).

La conversión del colesterol a coprostanol por *Eubacterium coprostanoligenes* es otro de los mecanismos en la regulación de los niveles de colesterol. Se ha descrito una asociación negativa significativa de la presencia de estas bacterias sobre C-LDL séricos (Li *et al*., 1998, Ren et al., 1996). Finalmente, el LPS, aunque su influencia es más significativa en la inducción de inflamación, se ha descrito su potencial papel en el metabolismo lipídico, aumentando los niveles de VLDL (Jaroonwitchawan *et al*, 2020, Schoeler y Caesar, 2019). Por todo lo anterior, debido a la implicación de la microbiota en el metabolismo del colesterol, se han propuesto enfoques terapéuticos para mejorar la composición y funcional de la microbiota intestinal, dentro de estos se incluye el consumo de alimentos con compuestos bioactivos.

1.2.4. Lácteos Fermentados como Coadyuante en el Manejo de la Hipercolesterolemia

Dado el papel de la hipercolesterolemia en el desarrollo de la aterosclerosis y las ECV, se han buscado estrategias para el manejo y regulación del colesterol, particularmente C-LDL. De acuerdo a las guías del manejo de lípidos, la integración de alimentos funcionales a la dieta debe ser un pilar básico del tratamiento no farmacológico de la hipercolesterolemia (Mach *et al.,* 2019). Los lácteos fermentados son un grupo de alimentos que han mostrado efectos benéficos a la salud cardiovascular de acuerdo con los recientes estudios sistemáticos y de metaanálisis. Estos estudios indicaron que dichos efectos benéficos se asociaron a su efecto en los niveles de C-LDL y triglicéridos. Por lo tanto, dichos estudios concluyeron que el consumo de estos productos (100g/día) puede ejercer un efecto clínico significativo (Ziaei *et al*., 2021, Pourrajab et al, 2020, Machlik *et al*., 2021).

Los productos lácteos fermentados son un grupo heterogéneo de alimentos, que difieren, principalmente, por los microorganismos empleados para la fermentación, y por ende en los nutrientes y metabolitos generados después del proceso fermentativo (Machlik *et al*., 2021). Los lácteos que han demostrado efecto hipocolesterolémico muestran que los microorganismos utilizados estuvieron en consorcio (*v.g.* kéfir), o empleadas como cultivos específicos en co-cultivo, múltiples cepas o mono cultivo. Algunos ejemplos de bacterias usadas en la preparación de leches fermentadas hipocolesterolemicas son *Bifidobacterium* (*Longun*, *animalis*), *Lactobacillus* (*v.g*. *rhamnosus* LV108, *Lactobacillus fermentum* grc08, MTCC:5898, *paracasei*, *plantarum*, *acidophilus* 1700) (Wa *et al*, 2019, Yadav, 2019, Chiu et al., 2006), *Pediococcus* (*acidilactici* BK0) (Melia *et al.,* 2023) y *Lactococcus* (*v.g*. *lactis* subs. *cremoris* STB049) (Nakajima *et al*., 1992). La mayoría de las bacterias empleadas fueron seleccionadas con base en sus características probióticas y sus propiedades hipocolesterolémicos probadas *in vitro* o *in vivo*. Se ha descrito ampliamente que cepas específicas de bacterias probióticas pueden reducir los niveles de colesterol por múltiples mecanismos que explican el efecto hipocolesterolémico, estos incluyen el metabolismo de las sales biliares, la captación de colesterol y la modulación de la microbiota intestinal (Hassan *et al*., 2019).

1.2.5. Modulación de la Microbiota Intestinal por Lácteos Fermentados y su Impacto en el Metabolismo del Colesterol

Las investigaciones sobre las interacciones entre huésped-microbiota, y los factores dietéticos es un concepto emergente que merece consideración. A este respecto, múltiples estrategias han sido investigadas con este fin, como lo es el consumo de probióticos, prebióticos y postbioticos (Ceapa *et al*., 2013). Los productos lácteos fermentados son ricos en estos componentes, por lo que se han realizado estudios para mejorar la estructura y función de la microbiota intestinal. Los resultados de estudios de intervención, en humanos y animales, indican que en el consumo de lácteos fermentados tiene un impacto positivo en la microbiota (Guillemard *et al*., 2021). En específico, algunos efectos de los lácteos fermentados sobre la microbiota son, el incremento de bacterias consideradas benéficas (*v.g*. *Lactobacillus*, *Rumminococcus*, *Akkermansia*, *Bifidobacterium*, *Lachnospiraceae*, *Blautia*, *Roseburia*) y el decremento en la abundancia de bacterias potencialmente patógenas (Guillemard *et al*., 2021, Wang *et al*., 2012, Veiga *et al*., 2014). Al mismo tiempo, se han observado cambios en la abundancia de AGCC, como lo es el incremento de butirato (Veiga *et al*., 2014, Berni *et al*., 2017, Yu *et al*., 2020). Los beneficios fisiológicos, como consecuencia de estos cambios, se reflejan en el mejoramiento de ciertos trastornos, como los son las enfermedades inflamatorias intestinales, neurodegeneración y desordenes metabólicos. A este respecto, leches fermentadas con bacterias acido lácticas específicas han demostrado potencial para modular la microbiota, y consiguientemente, mejorar el perfil de lípidos.

Por ejemplo, la administración de leche fermentada con *Lactobacillus rhamnosus* (hrsyfm 1301) mejoró los niveles de lípidos séricos y aumentó la abundancia de *Bacteroides* y *Verrucomicrobia*. Se observó también una correlación positiva entre *Dorea* spp. y colesterol total y C-LDL, mientras que *Butyrivibrio* spp. se asoció negativamente con colesterol total y LDL-C (Chen *et al*., 2014). Por otro lado, Harata *et al*., 2017 reveló que el consumo de leche fermentada con *Lactobacillus rhamnosus* GG y *Lactobacillus gasseri* TMC0356, en personas, mejoró la composición de la microbiota. Con ello, se observó una correlación positiva con C-LDL y C-HDL y *Bacteroide*s, mientras que *Firmicutes* se asoció negativamente con C-LDL y C-HDL. De manera similar, Jiang *et al*., 2022 mostró que la administración de leche fermentada con *Bifidobacterium longum* 070103,

en ratones, redujo los niveles de colesterol total y C-LDL. Estos cambios coincidieron con un aumento de la diversidad y una disminución de la relación *Firmicutes/Bacteroidetes*. Además, *Bifidobacterium*, *Rhodospirillales*, *Lachnospiraceae* y *Peptococcaceae* se asociaron negativamente con colesterol total y C-LDL. Recientemente, los hallazgos de Melia *et al*., 2023 revelaron que la administración leche fermentada con *Pediococcus acidilactici* BK01 en ratas redujo significativamente los niveles de colesterol total y triglicéridos, junto con un aumento significativo de la abundancia de BAL en el intestino delgado.

Uno de los mayores efectos por el consumo de lácteos fermentados es el incremento de AGCC a nivel intestinal. Por lo que este incremento puede explicar el efecto hipocolesterolémico de las leches fermentadas por la modulación de la microbiota intestinal. Como se mencionó anteriormente, los AGCC participan en el metabolismo del colesterol. Los diferentes estudios del consumo de leche fermentada muestran un aumento significativo de bacterias productoras de butirato en paralelo al aumento de la concentración de butirato (Guillemard *et al*., 2021, Veiga *et al*., 2014, Berni *et al*., 2017). Los AGCC pueden regular los niveles de colesterol por diferentes mecanismos. Particularmente el butirato, inhibe la síntesis de colesterol por la modulación de SREBP2. El butirato incrementa el eflujo del colesterol hacia el lumen intestinal mediante la expresión del transportador ABCG5/8 y activa CYP7A1, también se ha descrito se efecto inhibitorio sobre la expresión de NPC1L1. Sin embargo, a pesar de que existen números estudios que han estudiado el papel de los AGCC, son limitados los reportes que demuestren el papel de las leches fermentadas en la modulación del metabolismo del colesterol por la acción de AGCC o bien de ácidos grasos de cadena ramificada (AGCR).

1.2.6. Componentes de los Lácteos Fermentados y su Efecto en la Microbiota Intestinal y el Metabolismo del Colesterol

Muchos los estos estudios han empleado bacterias probióticas en la preparación de leches fermentadas hipocolesterolémicas. No obstante, se ha reportado que los productos lácteos fermentados pueden presentar propiedades funcionales independiente de la presencia de bacterias

viables en el organismo (González *et al*., 2019). En este sentido, Berni *et al*., 2017 reportó que el consumo de leche fermentada (*Lactobacillus paracasei* CBAL74), tratada térmicamente, aumentó la abundancia relativa de *Lactobacillus*, *Blautia*, *Roseburia* y *Faecalibacterium*. Lo anterior, fue consistente con aumento de butirato, así como un incremento de los genes asociados a su síntesis. La modulación de la microbiota por lácteos fermentados con bacterias no probióticas puede deberse a los componentes presentes. Durante la fermentación, la composición de la leche se modifica por la actividad metabólica de las bacterias. Lo anterior resulta en una diversidad de nutrientes, componentes y metabolitos bioactivos, tales como, los exopolisacáridos (EPS), hidrolizados proteicos o péptidos y AGCC (Berni *et al*., 2017).

Algunos lácteos fermentados se han caracterizado por una alta producción de EPS. En un estudio realizado por Nakajima *et al*., (1992) demostró que la leche fermentada por *Lactococcus lactis* subsp. *cremoris* SBT0495, caracterizada por producir EPS, redujo los niveles de colesterol sérico en ratas, en comparación con una leche fermentada sin este componente. El EPS, o bien, el complejo EPS-proteína (caseína) puede reducir los niveles de colesterol a través de la unión con sales biliares en el intestino (Zhang *et al*., 2021). Además, se ha descrito que el EPS puede ejercer efecto hipocolesterolémico a través de su función como prebiótico (Korcz *et al*., 2018). Se ha sugerido que, en el intestino, el EPS puede estimular selectivamente el crecimiento de bacterias. Lo anterior también puede resultar en el incremento de ACGG tras el metabolismo de los EPS (Oerlemans *et al*., 2021).

El componente proteico de la leche puede también modular la microbiota intestinal. Por ejemplo, la administración de glicomacropéptido (GMP) en ratones mejoró los niveles de AGCC, en paralelo con una reducción de *Desulfovibrio* (Sawin *et al*., 2015). Los hidrolizados o péptidos derivados de las proteínas de la leche han demostrado también mejorar la composición de la microbiota y mejorar indicadores metabólicos (Aloo y Oh, 2022). Li *et al*. (2019) demostró que la administración de hidrolizados de α-lactalbúmina, incrementó la relación *Bacteroidetes/Firmicutes*, así como la abundancia de *Lachnospiraceae* y *Blautia*. Asimismo, los hidrolizados de caseína atenuaron la dislipidemia, con un ligero decremento de C-LDL, en conjunto con el incremento de la relación *Bacteroides/Firmicutes*, y la abundancia de *Blautia*, *Allobaculum* y *Ruminiclostridium* (Yuan *et al*., 2020). Por otro lado, los péptidos de suero de leche mostraron

capacidad para estimular el crecimiento de *Lactobacillus acidophilus* y *Bifidobacterium animalis in vitro,* y mejoraron los niveles de *Lactobacillus* y Bacteroidetes *in vivo* (Ya-Ju *et al*., 2016). No obstante, péptidos o hidrolizados proteicos derivados de la fermentación de la leche por BAL aún no han sido explorados.

El efecto modulador de los hidrolizados sobre la microbiota puede explicarse por su función en el crecimiento selectivo de bacterias que metabolizan las proteínas (*v.g*. *Clostridia* y *Bacteroides*). Si bien, la modulación de la microbiota es el resultado principalmente de carbohidratos no digeridos, se han detectado AGGC y AGCR por la ingestión de proteínas e hidrolizados (Ashaolu, 2020). Por lo tanto, se ha sugerido el papel prebiótico de los hidrolizados proteicos al incrementar la proporción de bacterias probióticas (v.g. *Lactobacillus acidophilus* NCFM) (Feng *et al*., 2022). Considerando a los productos lácteos como un alimento heterogéneo, existe una gran diversidad de metabolitos que ejercen efecto benéfico a la salud. En cambio, el papel hipocolesterolémico de estos metabolitos no ha sido ampliamente investigado. No obstante, se ha descrito el potencial efecto hipocolesterolémico de algunos componentes, como son los EPS y los péptidos bioactivos (Kocz *et al*., 2018, Kawase *et al*., 2001).

1.2.7. Péptidos Hipocolesterolémicos y Mecanismos Subyacentes

Los péptidos bioactivos son capaces de reducir los niveles de colesterol por diferentes mecanismos. Su acción puede ser a nivel exógeno (lumen intestinal) o endógeno. En la **Figura 2** se ilustran los principales mecanismos de los péptidos hipocolesterolémicos. A nivel exógeno ocurre cuando los péptidos son ingeridos ya sea en hidrolizados o inactivos en la matriz proteica, pueden ser nuevamente hidrolizados por las enzimas gastrointestinales, lo que puede generar péptidos más pequeños y/o aminoácidos. En el lumen intestinal, los péptidos pueden ejercer su efecto durante la absorción del colesterol dietario o biliar en diferentes puntos del proceso (Boachie *et al*., 2018). Los péptidos pueden inhibir o desestabilizar las micelas de colesterol, lo que disminuye la absorción de colesterol (Zhang *et al.,* 2012). En el lumen intestinal los péptidos pueden inhibir la enzima colesterol esterasa (CE). La CE mejora la incorporación de colesterol a las micelas y en las

membranas del borde del cepillo. Por lo tanto, la inhibición de CE por péptidos puede impactar en el grado de absorción de colesterol (Fisayo *et al*., 2021, Zhao *et al*., 2022). Otra forma de reducir la absorción de colesterol por péptidos se da mediante el bloqueo del transportador NPC1L1 ubicado en el borde del cepillo (Real y Gonzalez, 2017). La capacidad de unión de péptidos a las sales biliares ocurre de manera similar a la colestiramina. Además, se ha demostrado que los péptidos pueden inducir la expresión de CYP7A1 independientemente de la interacción con sales biliares (Lee *et al*., 2021).

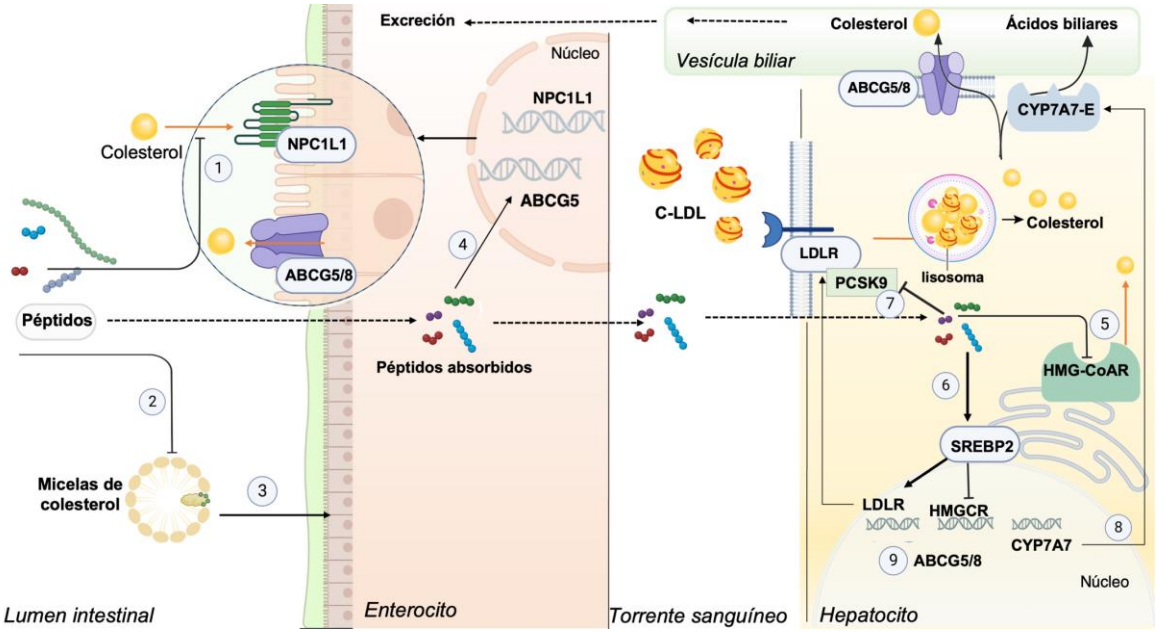

Figura 2. Mecanismos de la reducción de colesterol por péptidos bioactivos. (1) Inhibición competitiva de NPC1L1, (2) Inhibición de la formación de micelas de colesterol, (3) Unión a sales biliares, (4) Regulación de la absorción transepitelial de colesterol, (5) Inhibición enzimática de HMG-CoA reductasa, (6) Modulación del factor de transcripción SREBP2, (7) Inhibición de PCSK9, (8) Activación de la expresión de CYP7A7, (9) Activación de la expresión de ABCG5/8. (Fuente: elaboración propia en BioRender.com).

Los siguientes mecanismos (endógenos) requieren la absorción de los péptidos ya sea en los enterocitos o bien, cruzar la barrera intestinal para ejercer su acción a los hepatocitos. En los enterocitos, los péptidos pueden aumentar el eflujo del colesterol transepitelial a través de la inducción y supresión de ABCGG5/8 y NPC1L1, respectivamente. A nivel hepático, los péptidos bioaccesibles puede inhibir la síntesis de colesterol, a través de la inhibición de HMG-CoA

reductasa, lo que conduce a la disminución de colesterol por la captación de C-LDL y expresión de LDLR (Silva *et al*., 2021). La inhibición de la síntesis y aumento de LDLR también ocurre por la modulación de SREBP2 por péptidos específicos (Boachie *et al*., 2018). La actividad antagónica de los péptidos sobre PCSK9 también ha sido reportada, lo que se refleja en una mayor expresión de LDLR y menores niveles de C-LDL (Macchi *et al*., 2021). Finalmente, el mecanismo subyacente de algunos péptidos hipocolesterolémicos consiste en aumentar el eflujo de colesterol del hígado hacia la vesícula biliar, para su excreción en el intestino. Lo anterior se explica por la inducción de la síntesis de los transportadores ABCG5/8. Por lo anterior, un mayor eflujo indica una menor acumulación de colesterol hepático (Caponio *et al*., 2020).

1.2.8. Péptidos Bioactivos Hipocolesterolémicos de Origen Lácteo

Existen diferentes péptidos caracterizados con efecto hipocolesterolémico. Los péptidos caracterizados hasta el momento son heterogéneos en cuanto a su longitud y propiedades fisicoquímicas. El tamaño de estos péptidos comprende desde dipéptidos (*v.g*. prolina-fenilalanina, peso molecular: 262.12 Da) hasta 7 kDa. No obstante, el efecto de la longitud sobre el efecto hipocolesterolémico no está clara, ya que depende del mecanismo de acción. Por ejemplo, se ha reportado que los péptidos más activos para inhibir la solubilidad micelar del colesterol se encuentran en un rango de 0.3-0.8 kDa, mientras que para inhibir la HMG-CoA generalmente son menores a 0.3 Da. Aunado a esto, las propiedades como, la hidrofobicidad, el punto isoeléctrico y la carga neta parecen ser las características que mejor explican la actividad biológica (Zhang *et al*., 2012).

Existen diferentes matrices que han sido exploradas para la obtención de péptidos con función hipocolesterolémica. Los péptidos mayormente estudiados provienen de la proteína de soya (Kim *et al*, 2021). Sin embargo, se han caracterizado péptidos provenientes de otras fuentes proteicas alimentarias como lo son las proteínas lácteas. Dentro de estas, se han identificado péptidos hipocolesterolémicos, tanto en las proteínas séricas, así como en las caseínas. Algunos hallazgos indican que las proteínas séricas tienen mayor efecto reductor del colesterol que las caseínas. No

obstante, hallazgos recientes han evidenciado que péptidos específicos de caseínas parecen ejercer el efecto por múltiples mecanismos sobre la regulación del metabolismo del colesterol. Es importante considerar que, el grado de efectividad de los péptidos está en función de su estructura. Sin embargo, inicialmente, depende del método de liberación o hidrólisis (Hajfathaliam *et al*., 2018).

Para la liberación de péptidos hipocolesterolémicos derivados de las proteínas lácteas, en general, se han empleado enzimas específicas. De hecho, los reportes indican que la tripsina, pepsina y neutrasa fueron las mayormente empleados (Nagaoka *et al*., 2001, Jian *et al*., 2020, Lee *et al*., 2020). La fermentación por BAL proteolíticas es un método que se ha sido utilizado para la obtención de péptidos con múltiples funciones biológicas (*v.g*. antihipertensivo, antioxidante) (Samtiva *et al*., 2022). Cabe mencionar que hasta donde es de nuestro conocimiento, la fermentación de leche por BAL no ha sido empleada para la liberación de péptidos hipocolesterolémicos, pero si para otras bioactividades, a pesar del hecho de que varios estudios indicaron el efecto reductor de colesterol de leches fermentadas por BAL específicas. Para explicar el efecto hipocolesterolémico de las leches fermentadas, Kawase *et al*., (2001) aisló los componentes de una leche fermentada por *Streptococcus thermophilus* en función del peso molecular. La fracción de bajo peso molecular (< 10 kDa), fracción de alto peso molecular (> 10 kDa), en conjunto con el extracto total (suero) y la leche fermentada fueron evaluados en un modelo murino. Los hallazgos indicaron que la fracción de bajo peso molecular ejerció el mejor efecto hipocolesterolémico comparado con el resto de los tratamientos. Además, junto a este efecto, se observó una mayor excreción fecal de sales biliares y esteroides neutros, aunque no se identificaron los metabolitos asociados al efecto. Estudios posteriores pueden reforzar la hipótesis de que dicho efecto podría involucrar la acción de péptidos hipocolesterolémicos, particularmente, aquellos capaces de modular la absorción de colesterol y sales biliares.

En este respecto, un estudio realizado por Chen *et al*. (2007) mostró que hidrolizados proteicos derivados de una leche fermentada con cinco cepas de BAL presentaban capacidad de unión a sales biliares. Sin embargo, los péptidos responsables de la actividad biológica no fueron identificados. Adicionalmente, un estudio reciente exploró la actividad hipocolestrolémica de fracciones < 3kDa derivados de leches fermentadas con *Lactobacillus* spp. Las fracciones obtenidas tras un proceso

de digestión gastrointestinal simulada mostraron capacidad para reducir la formación de micelas de colesterol *in vitro* (Zambrano-Cervantes *et al*., 2023). En este sentido, Rendón-Rosales *et al*. (2019) investigó el potencial efecto hipocolesterolémico de fracciones peptídicas provenientes de leches fermentadas con *Lactococcus lactis* mediante estudios *in vitro*. Los hallazgos obtenidos mostraron que la fermentación de la leche con cepas de *Lactoccoccus lactis* NRR B-50572, NRRL B-50571 o NRRL B-50600 liberaban fracciones peptídicas capaces de inhibir la formación de micelas de colesterol y de unirse a las sales biliares. Por todo lo anterior, se hipotetiza un potencial efecto hipocolesterolemico *in vivo* de estas leches fermentadas. Lo anterior, también podría sustentar en parte, el efecto hipolipidémico observado anteriormente por Rodríguez-Figueroa *et al.* (2013).

A partir de estos estudios surge la presente tesis mediante la hipótesis por inducción. Según lo revisado en los antecedentes, el efecto hipocolesterolémico de las leches fermentadas es cepa dependiente y su efecto podría estar explicado por diferentes mecanismos que incluye la modulación de la microbiota intestinal, independientemente de la viabilidad de la bacteria (probiótica o no probiótica). También, el perfil de lípidos podría ser modulada directamente sobre el metabolismo del colesterol, ya sea exógeno o endógeno por los péptidos bioactivos. Sin embargo, son necesarios estudios en modelos *in vivo* con hipercolesterolemia para consolidar los hallazgos previos, especialmente con el uso de cepas de *L. lactis* específicas para la elaboración de leches fermentadas (Rodriguez-Figueroa et al., 2013) y con ello obtener una leche fermentada con capacidad para reducir el riesgo cardiovascular a través de la regulación de los niveles de lípidos.

1.3. Hipótesis

Leches fermentadas con cepas específicas de *Lactococcus lactis* presentan potencial efecto cardioprotector en modelos murino con hipercolesterolemia a través de la regulación de los niveles de lípidos por péptidos bioactivos y por la microbiota intestinal

1.4. Objetivo General

Evaluar el efecto hipocolesterolémico de leches fermentadas con cepas específicas de *Lactococcus lactis* en modelos murino mediante la regulación de los niveles de lípidos por péptidos bioactivos y por la microbiota intestinal.

1.5. Objetivos Particulares

1. Evaluar el efecto hipocolesterolémico de leches fermentadas con cepas de *L. lactis* específicas en modelo murino con hipercolesterolemia inducida.

2. Evaluar la respuesta de la microbiota intestinal por el consumo de leches fermentadas por cepas específicas de *L. lactis* en un modelo murino con hipercolesterolemia.

3. Evaluar la actividad hipocolesterolémica de fracciones péptidicas aisladas de leches fermentadas con *L. lactis* basado en mecanismos específicos *in vitro*.

4. Evaluar el efecto de la administración de fracciones solubles en agua derivadas de leches fermentadas con cepas específicas de *L. lactis* en la regulación de lípidos en un modelo murino con hipercolesterolemia e identificar los péptidos potencialmente hipocolesterolémicos.

1.6. Sección Integradora

Cepas específicas de *Lactococcus* (*L.*) *lactis* fueron utilizadas para preparar leches fermentadas con el fin de evaluar su potencial efecto en la reducción de riesgo cardiovascular mediante la regulación de los niveles de colesterol. Previamente, Rendón-Rosales *et al*., (2019) mediante estudios *in vitro*

investigó las propiedades cardiprotectoras (antitrombótica e hipocolesterolémica) de leches fermentadas con cepas de *L. lactis* (NRRL B-50571, NRRL B-5050572, NRRL B-598, NRRL B-50599 y NRRL B-50600). Los hallazgos demostraron que tres leches fermentadas con cepas de *L. lactis* presentaron potencial hipocolesterolémico por la capacidad de las fracciones peptídicas de inhibir la formación de micelas de colesterol y unirse a las sales biliares en un proceso de digestión gastrointestinal simulado. Por lo tanto, a partir de esos hallazgos se planteó el siguiente trabajo, de los cuales surgieron cuatro artículos originales y uno complementario, los cuales se describen a continuación.

De acuerdo con los hallazgos de Rendón-Rosales *et al.* (2019), se seleccionaron tres cepas de *L. lactis* NRRL B-50571, NRRL B-5050572 y NRRL B-50600 ya que presentaron mejores propiedades hipocolesterolémicas. Con base a lo anterior se formuló el objetivo 1 de la tesis, que consistió en el evaluar el efecto hipocolesterolémico *in vivo* de las leches fermentadas con dichas cepas de *L. lactis.* Para evaluar el efecto hipocolesterolémico de las leches fermentadas seleccionadas (FM-571, FM-572 y FM-600) se utilizó un modelo *in vivo* utilizando ratas Sprague-Dawley con hipercolesterolemia inducida con una dieta alta en colesterol. La administración de las leches fermentadas junto con una leche sin fermentar (LSF) se realizó durante 7 semanas. Al final del periodo experimental se analizó el perfil de lípidos (colesterol total, C-LDL, C-HDL, C-VLDL y triglicéridos). Con base en estos parámetros de lípidos en plasma y el riesgo cardiovascular (índice aterogénico en plasma y coeficiente aterogénico), se observó que las leches fermentadas FM-572 y FM-600 mostraron reducir significativamente los niveles de colesterol total y reducir por consiguiente el índice aterogénico. También, se observó que, estas leches reducían los niveles de lípidos en hígado, y aumentó la excreción de lípidos totales en heces. Con ello se logró cumplir el objetivo 1 y los resultados se encuentran en el **artículo 1** (Rendón-Rosales et al., 2023).

Con base en los resultados anteriores, se formularon las siguientes interrogantes ¿Cuáles son los efectores de la reducción de colesterol por las leches fermentadas?, ¿La microbiota intestinal podría estar implicada en la regulación de lípidos?, y además ¿Los péptidos bioactivos derivados de la fermentación podrían ejercer efecto hipocolesterolémico?. Para responder estas preguntas, se analizó, en primer lugar, la respuesta de la microbiota intestinal por el consumo de las leches fermentadas. Basándonos en una estrategia de secuenciación masiva del gen 16SARNr, se analizó

la composición bacteriana a nivel colónico y fecal. Debido a que el metabolismo de lípidos está influenciado, principalmente por AGCC, se analizó también la concentración de AGCC (acetato, propionato y butirato) en heces. Esto con él propósito de conocer la asociación entre la microbiota intestinal y sus principales metabolitos (ACGG) con los parámetros lipídicos.

Como resultados de esta etapa (objetivo 2), se encontró que la respuesta en la composición de la microbiota por el consumo de las leches fermentadas fueron detectados a nivel de familia. Específicamente se observó una marcada diferencia entre el grupo sano y los grupos hipercolesterolémicos en la estructura de la microbiota intestinal, junto con un significativo decremento de los AGCC, principalmente de butirato. No obstante, el consumo de las leches fermentadas, específicamente con FM-572, logró incrementar la abundancia de bacterias consideradas benéficas. Con ello, se mejoraron los niveles de AGCC comparado con el consumo de una leche sin fermentar. Los análisis de correlación mostraron que, familias como *Ruminococcaceae*, y *Lactobacillaceae* fueron asociadas negativamente con el colesterol total y C-LDL y positivamente con C-HDL. De igual forma, de los todos los AGCC, solo el butirato se correlacionó negativamente con colesterol y C-LDL, y positivamente con C-HDL. Por lo tanto, estos hallazgos indican que la microbiota intestinal está implicada en el efecto hipocolesterolémico de las leches fermentadas. Los resultados de esta etapa del estudio forman parte del **Manuscrito 2.**

Posteriormente, para contestar la interrogante de la implicación de los péptidos sobre el efecto hipocolesterolémico, que corresponde al objetivo 3, se planteó, primero, una estrategia que implicaba evaluar la bioaccesibilidad y biodisponilidad de péptidos de acuerdo a las actividades biológicas estudiadas. A este respecto, la inhibición de la formación de micelas de colesterol se estudió en fracciones peptídicas aisladas y purificadas provenientes de leches fermentadas (FM-572 y FM-600) sometidas a digestión gastrointestinal. Posteriormente, surgió la interrogante si las fracciones peptídicas también podrían tener implicación sobre la biosíntesis de colesterol. En este sentido, debido a que la biosíntesis de colesterol se lleva a cabo intracelularmente (hepatocitos o enterocitos), se realizó un experimento que involucraba la absorción de péptidos *ex vivo* usando intestino (yeyuno) de rata Wistar. Los hallazgos consolidaron los resultados obtenidos en la primera etapa, indicando que, fracciones peptídicas presentaban capacidad para inhibir la formación de micelas de colesterol. Los resultados en esta etapa además evidenciaron que la fracción peptídica

en un rango de peso molecular de 0.29 a 0.62 kDa presentó la mejor eficiencia de inhibición. Por otro lado, los péptidos absorbidos *ex vivo* mostraron capacidad para inhibir la HMG-CoA reductasa. No obstante, la fracción permeada de FM-600 digerida, mostró la mejor eficiencia inhibitoria, a pesar de que los péptidos, fueron los menos absorbidos. Los resultados detallados se encuentran en el **manuscrito 3**

Con las etapas anteriores se evidenció que las leches fermentadas ejercían efecto hipocolesterolémico a través de la modulación de la microbiota intestinal y la posible participación de fracciones peptídicas. Sin embargo, siguiendo con la interrogante sobre la participación de las fracciones peptídicas, se planteó el objetivo 4. Para ello, se desarrolló un bioensayo para evaluar el efecto hipocolesterolémico de las fracciones de bajo peso molecular (F< 10 kDa) provenientes de las leches fermentadas FM-572 y FM-600 en un modelo *in vivo* con hipercolesterolemia inducida. Los hallazgos indicaron que la fracción de bajo peso molecular de ambas leches fermentadas mostraron efectos significativos sobre el mejoramiento del perfil de lípidos en ratas. No obstante, la F < 10 kDa de FM-572 mostró el mejor efecto al disminuir los niveles de triglicéridos, ácidos grasos libres (NEFA) y el riesgo cardiovascular. Este experimentó también reforzó la idea de que la inhibición de la formación de micelas de colesterol (artículo 1) podría estar implicado como un mecanismo subyacente al efecto, ya que en este experimento se observó una mayor excreción de colesterol fecal. Por lo tanto, el estudio se complementó con la identificación parcial de péptidos con capacidad para inhibir la solubilidad micelar del colesterol provenientes de F<10 kDa de FM-572. Estos resultados se presentan en el **manuscrito 4.**

La tesis se complemente con la exploración de las actividades biológicas de secuencias específicas de péptidos bioactivos derivadas de leches fermentadas con *L. lactis* NRRL B-50571 o NRRL B-50572. Los resultados muestran un potencial efecto benéfico en la salud cardiometabólica de los péptidos bioactivos al presentar actividades inhibitorias sobre las enzimas trombina, dipeptidil-peptidasa IV (DPP-IV) y la enzima convertidora de angiotensina (ECA). Además, estos hallazgos demuestran que la fermentación por BAL, específicamente con *L. lactis* puede generar péptidos multifuncionales. Estos resultados se describen en el **artículo 5** (Rendón-Rosales et al., 2021).

2. HYPOCHOLESTEROLEMIC EFFECT OF MILKS FERMENTED WITH SPECIFIC STRAINS OF *Lactococcus lactis* IN HYPERCHOLESTEROLEMIC SPRAGUE-DAWLEY RATS

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Article

Hypocholesterolemic Effect of Milks Fermented with Specific Strains of *Lactococcus lactis* in Hypercholesterolemic Sprague−Dawley Rats

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ABSTRACT: This study assessed the hypocholesterolemic effect of fermented milks (FMs) with *Lactococcus lactis* NRRL B-50572 (FM-572) and NRRL B-50600 (FM-600) in a hypercholesterolemic rat model. Results indicated that FM-572 and FM-600 significantly (p < 005) decreased total plasma cholesterol in a range of 13 to 20% and non-HDL-C in a range of 20 to 23%. Therefore, both fermented milks significantly reduced the atherogenic coefficient (non-HDL-C/HDL-C ratio) and the atherogenic index of plasma in comparison with nonfermented milk. Liver lipids (total lipid, triglycerides, and cholesterol) were decreased, specifically with FM-600, compared to nonfermented milk. The atherogenic index was positively associated with the hepatosomatic index and negatively associated with HDL-C levels in plasma (p < 0.05). Excretion of cholesterol and bile acids was not associated (p > 0.05) with the cholesterol-lowering effect of these fermented milks. Overall, these findings support the potential hypocholesterolemic effect of fermented milks against cardiovascular diseases.

KEYWORDS: *fermented foods, hypercholesterolemia, cardioprotective effect, lactic acid bacteria, cholesterol metabolism, Lactococcus lactis, cholesterol lowering effect*

1. INTRODUCTION

Elevated serum cholesterol, particularly low-density (LDL-C) and very low-density cholesterol (VLDL-C), is the risk factor involved in atherosclerosis pathology, leading to the development of cardiovascular diseases (CVDs) which is the major cause of mortality worldwide.[1] The increase of these types of lipoproteins, coupled with the reduction of high-density lipoprotein cholesterol (HDL-C), is caused by diverse factors[2,3] (e.g., poor nutritional diet, genetic or familial hypercholesterolemia) and alteration of gut microbiota.

Particularly, the alteration of gut microbiota structure and function results in dysregulation of microbial metabolites such as short-chain fatty acids (SCFAs), secondary bile acids, and trimethylamine *N*-oxide. Changes in these metabolites can result in the alteration of cholesterol and bile acid metabolism, increasing LDL-C levels. Also, dysbiosis is associated with inflammation, oxidative stress, hypertension, and nonalcoholic fatty liver disease (NAFLD). Collectively, these alterations conduct the development of cardiometabolic diseases.[4,5]

The current therapeutic strategies are based on drugs that reduce cholesterol synthesis (HMGCR inhibitors), intestinal cholesterol absorption (NPC1L1 inhibitors), and excretion of bile acids by bile acid sequestrants such as cholestyramine.[6] Besides, lifestyle intervention is also an approach elected to improve plasma lipid profiles according to guidelines for the management of dyslipidemia.[7] In this sense, healthy nutrition such as functional food consumption is recommended to improve cardiovascular health.[8,9] In this regard, meta-analysis of cohort studies has evidenced that consumption of fermented dairy products with probiotic or nonprobiotic bacteria significantly decreased CVD risk. The cardioprotective effect of fermented milks is related to the decrease of blood pressure and the decrease of triglycerides and cholesterol levels in the bloodstream.[10,11] Thus, the development of dairy functional foods is important since they may act as adjuvants in lipid management.[12] Most studies reveal that the hypocholesterolemic effect is due to the presence of specific strains of probiotics in these products. Hence, living microorganisms in the gastrointestinal tract are necessary for this effect to be exerted.[13,14] The mechanisms behind the effect of these microorganisms were attributed to gut microbiota regulation, liver function improvement (by improving liver enzymes), and the capacity of probiotics to metabolize cholesterol or bile acids. A detailed description of these mechanisms may be found in previous reviews.[15−17]

It is noteworthy to mention that the hypocholesterolemic effect of fermented milks can also be due to specific nonprobiotic lactic acid bacteria, and this effect may be related to metabolites released during fermentation.[18] In this sense, milks fermented with *Lactococcus lactis* NRRL B-50571 and NRRL B-50572 improved lipid levels (reduction of serum LDL-C and triglycerides) in normocholesterolemic rats and hypertensive subjects.[19,20] Due to the high proteolytic activity

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Table 1. Clinical Characteristics of Sprague-Dawley Rats[a]

Experimental groups	SD	HCD	nFM	FM-571	FM-572	FM-600	*p*-value
Plasma TC baseline (mg/dL)	62.2 ± 8.0a	64.0 ± 8.6a	62.1 ± 7.9a	64.3 ± 11.6a	63.6 ± 8.2a	62.4 ± 7.2a	0.99
Body weight baseline (g)	269.8 ± 15.6a	267.5 ± 17.7a	278.1 ± 3.7a	267.8 ± 14.6a	269.0 ± 25.4a	268.4 ± 17.1a	0.86
Plasma TG baseline (mg/dL)	95.15 ± 12a	88.4 ± 21a	93.1 ± 16a	78.6 ± 24a	80.1 ± 8.5a	78.7 ± 8.1a	0.32

[a]Data represents the mean of independent biological replicates (n = 5 for SD group and n = 6 for high cholesterol diet groups) ± standard deviation. TC, total cholesterol; TGs, triglycerides.

reported of these *Lactococcus lactis* strains in lactic proteins, it was hypothesized that peptide fractions are potentially responsible for the cholesterol lowering effect. Afterward, *in vitro* studies demonstrated that *Lactococcus lactis* NRRL B-50571, NRRL B-50572, and NRRL B-50600 release peptide fractions capable of decreasing cholesterol micellar solubility and bile acid binding capacity. Additionally, these bioactivities were maintained after simulated gastrointestinal digestion of fermented milks.[21] It is important to highlight those fermented milks with these strains, specifically with NRRL B-50571 and NRRL B-50572, were reported to exert antihypertensive and heart rate-lowering effects, improve antioxidative status, and present antithrombotic activity.[21−23] Altogether, the multifunctional properties of these fermented milks may help to improve cardiovascular health. Therefore, the aim of this study was to evaluate the effect in the regulation of lipid levels in plasma and liver and lipid excretion by the consumption of fermented milks with *Lactococcus lactis* NRRL B-50571, NRRL B-50572, and NRRL B-50600 in an *in vivo* model.

2. MATERIALS AND METHODS

2.1. Bacterial Strains, Chemicals, and Diets. *Lactococcus (L.) lactis* NRRL B-50571, NRRL B-50572, and NRRL B-50600 were obtained from the Culture Collection of the Dairy Laboratory at the Food Research and Development Center, A.C. (Spanish acronym, CIAD, A.C., Hermosillo, Sonora, México). M17 broth, microbiology grade lactose, and dextrose were purchased from Difco (Sparks, MD). Total cholesterol, LDL/VLDL, and HDL cholesterol assay kits were purchased from Cell Biolabs (San Diego, CA). Triglycerides and glucose assay kits were purchased from Randox Laboratories (Crumlin, U.K.). A bile acid assay kit, cholic acid, and cholesterol were purchased from Sigma-Aldrich (Saint Louis, MO). Laboratory rodent diet (LabDiet 5001) was obtained from Pet Foods (México City, México).

2.2. Preparation of Fermented Milks. *L. lactis* strains were cultivated in M17 broth supplemented with a lactose or dextrose solution (10% v/v) in concordance with a previous report. Skim cow's milk was used for preparing the preinoculum in milk and in fermented milks. Preinoculums in milk were prepared in heat-treated milk (110 °C/10 min) at 3% v/v of each bacteria culture and incubated at 30 °C for 12 h for NRRL B-50571 and NRRL B-50572, whereas the preinoculum of NRRL B-50600 was incubated for 24 h. Fermented milks (FMs) were prepared using nonfat reconstituted milk (10% w/v) and incubated at 30 °C for 48 h. The fermentation process was stopped with heat treatment (75 °C/15 min).[21,22]

2.3. Animals and Experimental Treatments. A total of 35 male Sprague−Dawley rats (271 ± 16 g body weight; 6 weeks old) were obtained from the Experimental Laboratory of the University of Sonora (Hermosillo, Sonora, México). Rats were maintained in individual cages with *ad libitum* intake of a commercial rodent standard diet (LabDiet 5001, Bioinvert, Mexico City, Mexico) and purified water. The environmental conditions were regulated at 21 ± 2 °C, 45−60% relative humidity, and 12-h light-dark cycles (06:00−18:00). After acclimatization for 3 weeks, the rats (n = 35) were randomized and divided into six groups (five groups of n = 6 and one group of n = 5) using plasma total cholesterol (TC) and body weight as variables for randomization (p > 0.95). Afterward, the following treatments were assigned to the groups. 1) Standard diet group (SD), 2) high-cholesterol diet (HCD) that consisted of 98.75% of a standard diet, 1% w/w cholesterol, and 0.25% w/w cholic acid, 3) HCD + nonfermented milk (nFM), 4) HCD + FM with *L. lactis* NRRL B-50571 (FM-571), 5) HCD + FM with *L. lactis* NRRL B-50572 (FM-572), and 6) HCD + FM with *L. lactis* NRRL B-50600 (FM-600). All fermented milks and nonfermented milk were administered *ad libitum* for 7 weeks. During this period, milk and food consumption as well as body weight were measured. The animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Bioethics Committee of the CIAD (CE/017/2019).

2.4. Biological Sample Collection. Animals were deprived of food for 12 h, and then they were euthanized by anesthesia administration (pentobarbital) followed by intraperitoneal injection (75 mg/kg of body weight). Blood samples were obtained by intracardiac puncture and collected in heparinized tubes. For plasma collection, blood was centrifuged (2500*g*, 15 min, 4 °C). Fresh feces were collected at the end of the experimental period. The liver was removed, weighed, and stored at −80 °C, and the hepatosomatic index was calculated as follows:[24]

Hepatosomatic index = (liver weight/body weight) × 100

2.5. Biochemical Analysis in Plasma. Total cholesterol (TC), non-HDL-C, and HDL-C levels were measured in heparinized plasma using fluorometric enzymatic kits. Triglycerides (TG) and glucose were quantified using colorimetric enzymatic kits. The atherogenic index of plasma and the atherogenic coefficient were calculated by using previously reported equations.[25] Non-HDL-C was defined as LDL-C + VLDL-C

$$\text{Atherogenic coefficient (AC)} = \text{non-HDL-C/HDL-C}$$

$$\text{Atherogenic index of plasma (AIP)} = \log[\text{TGS (mM)/HDL-C (mM)}]$$

2.6. Lipid Content in Liver and Feces. Total lipid content was determined in liver tissue and freeze-dried feces according to the modified Folch method.[26] 200 mg of sample (liver or feces) was homogenized with 10 mL of chloroform:methanol (2:1) for two min using an automatic homogenizer (Bio-Gen Pro2000 Homogeneizer, ProScientific, Oxford, CT). The homogenized samples were sonicated for five min and incubated overnight in an orbital water bath shaker (450 rmp, 25 °C). Then, samples were centrifuged (3000*g*, 10 min), and the supernatant was collected and washed with 2.5 mL of 0.9% NaCl (2500*g*, 10 min). The upper phase was discarded, and the lower phase was washed twice with 2.5 mL of 50% methanol (2500*g*, 10 min). The organic phase was recovered and dried with a stream of nitrogen in a water bath (45 °C). Total lipids were determined by the weight difference. For the enzymatic determination of lipids (TC and TG), lipids were reconstituted in a mixture (1:1 v/v) of isopropanol and triton X-100 (1% v/v).

2.7. Total Bile Acid Determination in Feces. Total bile acids were extracted from freeze-dried feces with minor modifications.[27] A 100-mg portion of feces was homogenized with 1 mL of absolute ethanol and subsequently sonicated for 10 min. The homogenized sample was heated at 60 °C for 30 min and subsequently cooled to 25 °C. The mixture was then centrifuged (1600*g*, 10 min, 15 °C), and the supernatant was recovered. The pellet was washed twice with absolute ethanol and centrifuged at 11,200*g* for 1 min. The collected supernatants were mixed and diluted in water, and the bile acids were quantified using a fluorometric enzymatic assay kit.

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Table 2. Effect of Treatments on Plasma Total Cholesterol, Glucose, and Food, Milk, and Milk Protein Intake during the Experimental Period[a]

Experimental groups	SD	HCD	nFM	FM-571	FM-572	FM-600
Plasma TC (mg/dL) at week 3	73.1 ± 6a	134.3 ± 13b	122 ± 15b	132.5 ± 21b	119.2 ± 11b	126.32 ± 6b
Plasma glucose (mmol/L) at week 7	6.43 ± 0.53a	6.39 ± 0.6a	6.25 ± 0.7a	6.68 ± 0.57a	6.25 ± 0.43a	6.52 ± 0.8a
Daily food intake (g/100 g body weight)	5.91 ± 0.55a	6.88 ± 0.61a	6.71 ± 0.75a	6.74 ± 0.64a	6.74 ± 0.66a	6.57 ± 0.51a
Daily FM or nFM intake (mL/100 g of body weight)	-	-	4.36 ± 0.82a	4.09 ± 0.47a	5.06 ± 0.57a	4.06 ± 0.54a
Daily milk protein intake (mg/100 g of body weight)	-	-	122.5 ± 23[ab]	115.19 ± 13.21a	143.1 ± 16b	113.68 ± 15a

[a]Data represents the mean of independent biological replicates (n = 5 for standard diet group n = 6 for high cholesterol diet groups) ± standard deviation. Different letters indicate statistical differences ($p < 0.05$) between the different experimental groups. Milk protein was determined by the Kjeldahl method (AOAC: 2.062).

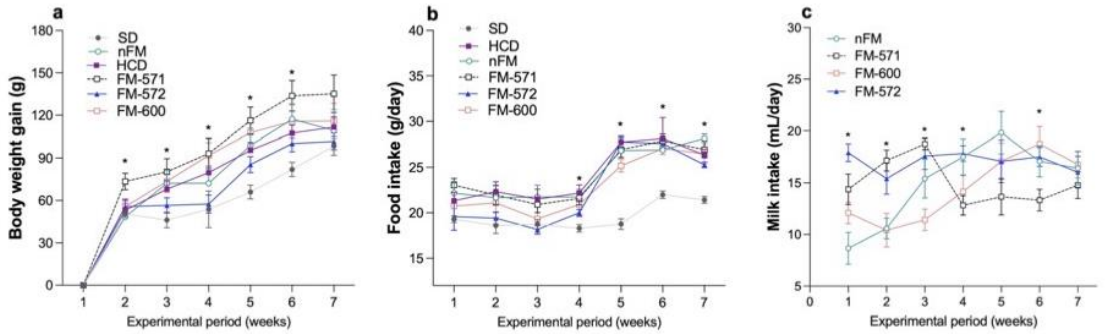

Figure 1. Effect of fermented milks with *L. lactis* on a) body weight gain, b) food intake, and c) milk intake on Sprague−Dawley rats with a high cholesterol diet for 7 weeks of treatments. Data represent the mean of independent biological replicates (n = 5 for standard diet group, n = 6 for high cholesterol diet groups) ± standard deviation. Asterisks (*) indicate statistical differences ($p < 0.05$) between experimental groups.

Table 3. Effect of Nonfermented Milk and Milks Fermented with *L. lactis* on the Plasma Lipid Profile and Cardiovascular Risk Indexes in Sprague−Dawley Rats with a High Cholesterol Diet at the End of the Experimental Period (Week Seven)[a]

Experimental groups	SD	HCD	nFM	FM-571	FM-572	FM-600
TC (mg/dL)	88.04 ± 9.16a	138.84 ± 10.37b	145.41 ± 27.99b	123.84 ± 28.03bc	109.39 ± 18.47ac	119.78 ± 21.0bc
Non-HDL-C (mg/dL)	37.36 ± 6.38a	114.56 ± 13.95b	114.29 ± 22.13bc	99.80 ± 23.13bc	90.71 ± 16.06cd	87.72 ± 18.76cd
HDL-C (mg/dL)	52.98 ± 5.49a	33.71 ± 3.14bc	29.43 ± 8.21b	31.13 ± 6.89b	34.71 ± 4.54bc	40.66 ± 1.34c
TG (mg/dL)	100.86 ± 2.52a	115.29 ± 9.96a	144.43 ± 20.20b	107.75 ± 11.27a	122.41 ± 14.92ba	117.30 ± 8.92a
AC (non-HDL-C/HDL-C)	0.71 ± 0.14a	3.42 ± 1.32b	4.74 ± 1.32c	3.78 ± 1.17b	2.46 ± 0.68d	2.16 ± 0.48d
AIP log (TGS/HDL-C)	−0.20 ± 0.03a	0.089 ± 0.02b	0.26 ± 0.07c	0.13 ± 0.05b	0.09 ± 0.03b	−0.04 ± 0.02d

[a]The data represent the mean of independent biological replicates (n = 5 for standard diet group n = 6 for high cholesterol diet groups) ± standard deviation. Different letters indicate statistical differences ($p < 0.05$) for the different experimental groups.

2.8. Experimental Design and Statistical Analysis. The data represents the mean of the independent biological replicates (n = 5 for the standard diet group, and n = 6 for the high cholesterol diet groups) ± standard deviation. A completely randomized design was used, and the normality test (Shapiro-Wilk test) was performed before the one-way analysis of variance (ANOVA) was carried out. Means were compared by using Fisher test. Differences were considered significant when $p < 0.05$. Analysis was performed using GraphPad Prism version 9.3.1 for iOS, GraphPad Software, San Diego California USA, www.graphpad.com. Correlation analysis and visualization were performed using the R-package corrplot via RStudio version 2021.09.2 using the Pearson coefficient.

3. RESULTS

3.1. Baseline Parameters and Effect of Fermented Milks in Body Weight, Food Intake, and Glucose. Baseline data of clinical characteristics of rats are displayed in Table 1. As expected, due to randomization, at the beginning of the experimental period, rats were statistically equal in total cholesterol, triglycerides, and body weight variables ($p > 0.8$). Henceforth, blood sampling was carried out on the third week in order to assess total cholesterol in plasma and oversee hypercholesterolemia. As shown in Table 2, total cholesterol in plasma increased near 2-fold with respect to the control (SD) ($p < 0.05$). At this point, the consumption of fermented milks with *L. lactis* did not show any significant effect on total cholesterol levels in plasma ($p > 0.05$). At the end of the experimental period (7 weeks), the rats were euthanized, and the biochemical parameters were evaluated. Glucose levels in plasma were not altered by the effect of a high cholesterol diet, as well as the consumption of milks fermented with *L. lactis* ($p > 0.05$) as observed in Table 2.

On the other hand, Table 2 summarizes the relative average food intake and amount of milk ingested. Despite the milks being administered *ad libitum*, the volume of milk and the dairy protein ingested were determined. The relative consumption of food and the different milks were statistically equal in all treated experimental groups ($p > 0.05$). Considering the total protein concentration in milks, protein from FM-572 was the most ingested as shown in Table 2 ($p < 0.05$).

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Furthermore, Figure 1 illustrates the behavior of body weight gain by the effect of the high-cholesterol diet and the consumption of the different milks for 7 weeks of treatments. Overall, the control group with the standard diet showed the lowest body weight gain. Conversely, FM-571 showed an increase of body weight, specifically these differences were found in the second to the sixth week of treatments ($p < 0.05$). Food and milk consumption were also recorded. As expected, food intake (Figure 1b) increased during the experimental period. Groups with a high cholesterol diet showed a higher consumption of food, specifically in weeks four to seven ($p < 0.05$). With respect to milk consumption (Figure 1c), differences in the milk volume ingested may be due to the way of administration. Overall, FM-572 and FM-571 were mostly consumed in the first weeks of the experimental period. On the contrary, nFM and FM-600 were the least consumed by the rats. Nevertheless, in the last week, the volume of milk ingested was the same for all treated groups ($p > 0.05$).

3.2. Effect of Consumption of Fermented Milks on the Plasma Lipid Profile and Atherogenic Indexes. To investigate the hypocholesterolemic effect of fermented milks with *L. lactis*, the plasma lipid profile in rats with a high-cholesterol diet was evaluated, and the cardiovascular risk through the atherogenic indexes was estimated. Table 3 summarizes the effect of fermented milk on lipid levels at the end of the experimental period. Total cholesterol levels were significantly higher in HCD and nFM groups ($p < 0.05$). Conversely, the FM-572 group showed lower levels (−21.2%) of total cholesterol ($p < 0.05$). In fact, no significant changes were observed between FM-572 and SD groups ($p > 0.05$). Also, FM-571 and FM-600 slightly reduced cholesterol levels ($p > 0.05$). Likewise, Non-HDL-C was 3-fold more in HCD and nFM groups compared with the SD group. FM-572 and FM-600 decreased by 21% and 23% non-HDL-C with respect to HCD ($p < 0.05$). With respect to FM-571, no significant changes were observed ($p > 0.05$). HDL-C was remarkably decreased in all HCD groups ($p < 0.05$), and no effect was found with the administration of fermented milks ($p > 0.05$). However, FM-600 slightly enhanced HDL-C levels. Furthermore, the atherogenic coefficient and AIP were increased in HCD and nFM groups ($p < 0.05$) with respect to the SD group. Conversely, FM-600 and FM-572 groups showed lower levels of atherogenic coefficients and AIP values ($p < 0.05$) compared with HCD and nFM groups.

Correlation analysis (Figure 2) indicated that HDL-C was the main variable associated with decreased AIP ($p < 0.05$). Furthermore, triglycerides were slightly increased after consumption of a high cholesterol diet; however, the more notable effect was the increase of triglycerides in the nFM group. Fermented milks did not exert a triglyceride lowering effect. Additionality, it was observed that total cholesterol levels were slightly decreased in week seven with respect to week three (Table 2) in groups with fermented milks, whereas in the nFM group, total cholesterol was increased. Collectively, these findings indicate that FM-572 and FM-600 exert beneficial effects in the reduction of cardiovascular risk through their hypocholesterolemic effect.

3.3. Effect of Consumption of Fermented Milks on Liver and Fecal Lipids and Bile Acids. We next investigated the lipid content in hepatic tissue (Figure 3a-e). At the hepatic level, all groups that received the hypercholesterolemic diet showed an increase of the hepatosomatic index ($p < 0.05$). Correlation analysis (Figure 2) indicated that the changes in

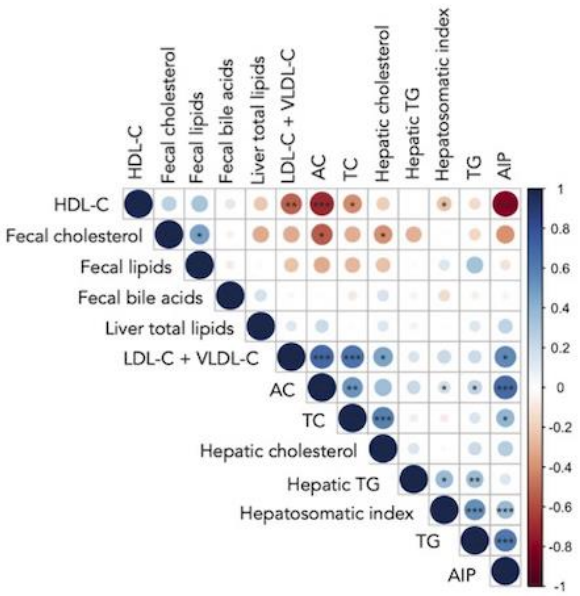

Figure 2. Heat map based on a dot plot representation of the correlation analysis that indicates the association between variables related to lipid metabolism. The size of each point represents the correlation coefficient, and the color represents a positive (blue) or negative (red) relationship. Asterisks represent statistical significance (* < 0.05, ** < 0.01, and *** < 0.0001).

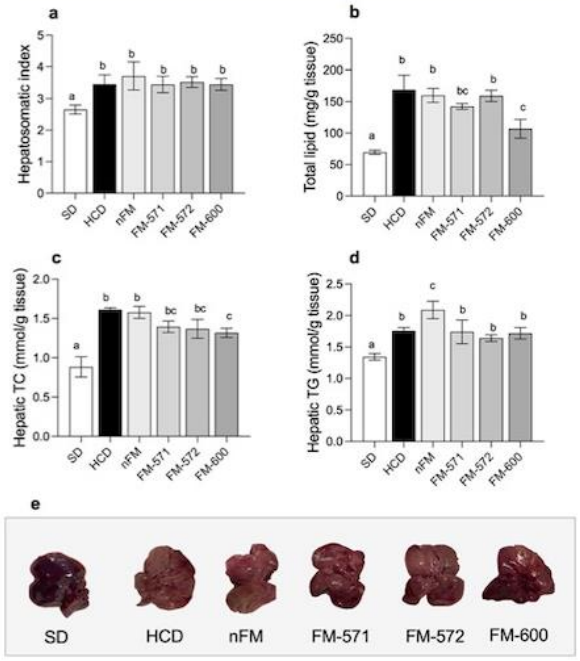

Figure 3. Effect of milks fermented with specific strains of *L. lactis* on liver parameters: a) hepatosomatic index, b) total lipids, c) total cholesterol (TC), d) hepatic triglycerides (TGs), and e) macroscopic appearance of liver. Data represents the mean of independent biological replicates ($n = 5$ for standard diet group; $n = 6$ for high cholesterol diet groups) ± standard deviation. Different letters indicate statistical differences ($p < 0.05$) between groups.

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the hepatosomatic index were significantly associated with triglycerides but not with cholesterol levels ($p < 0.05$). Also, the increase of the hepatosomatic index was positively associated with the atherogenic index but negatively associated with HDL-C ($p < 0.05$). Also, Figure 3 depicts the effect of fermented milks on hepatic lipids. Total lipids in liver (Figure 3b) were significantly increased in all groups with the high cholesterol diet ($p < 0.05$). However, total lipids in the FM-600 group were 25% less than the hypercholesterolemic group ($p < 0.05$). Likewise, hepatic cholesterol (Figure 3c) was increased in HCD ($p < 0.05$); nonetheless, the consumption of fermented milks, particularly with FM-600, decreased hepatic cholesterol 20% compared with HCD ($p < 0.05$). Moreover, hepatic triglycerides (Figure 3d) were also increased in all groups with a high-cholesterol diet ($p < 0.05$) and specifically in the nFM group. However, triglycerides were 16−21% lower in groups treated with fermented milks compared with nFM ($p < 0.05$). Altogether, increases of lipid levels apparently led to steatosis development, which could be associated with changes in the macroscopic liver appearance observed in this study (Figure 3e).

Furthermore, excretion of lipids was also determined; Figure 4 illustrates the effect of fermented milks on total lipids, cholesterol, and bile acid excretion. Total lipids in feces (Figure 4a) were increased in all groups with the high cholesterol diet. Specifically, an increase of total lipids excretion was found in the FM-572 group in comparison with HCD ($p < 0.05$). Fecal cholesterol excretion was higher in the hypercholesterolemic groups ($p < 0.05$). Nevertheless, no differences were detected in cholesterol excretion by the consumption of the fermented milks compared to the HCD group ($p > 0.05$). Besides, bile acid excretion was markedly higher in the hypercholesterolemic groups ($p < 0.05$), but no significant changes were observed with the administration of the different fermented milks ($p > 0.05$). In addition, correlation analysis (Figure 2) showed that neither cholesterol nor bile acid excretion was associated with cholesterol levels in plasma ($p > 0.05$).

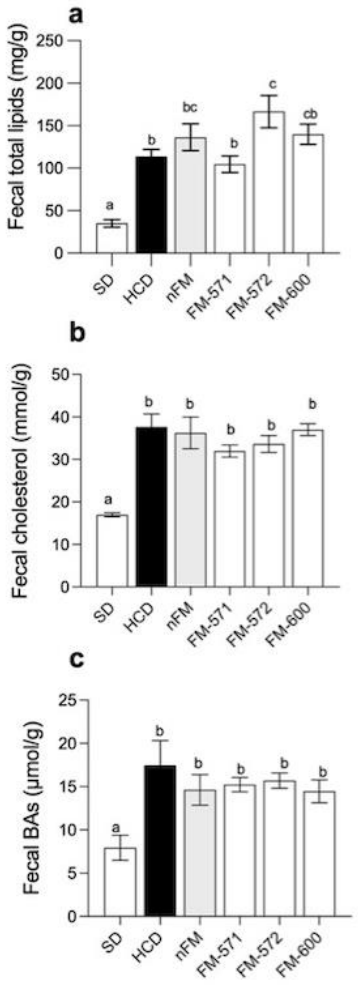

Figure 4. Effect of milks fermented with *L. lactis* on fecal excretion of a) total lipids, b) cholesterol, and c) bile acids (BAs) in Sprague−Dawley rats. Data represent the mean of independent biological replicates ($n = 5$ for standard diet group; $n = 6$ for high cholesterol diet groups) ± standard deviation. Different letters indicate statistical differences ($p < 0.05$).

4. DISCUSSION

Over the last decades, the increase of cardiometabolic diseases has attracted attention for the development of functional foods with cardioprotective properties. Fermented milk products were suggested as coadjuvants for lipid management and decrease dyslipidemia. Systematic studies reveal that the consumption of fermented milks reduces LDL-C.[12,10] According to dietary guidelines the consumption of fermented milks should be fomented as part of the diet.[7] In this regard, in the present study, an *in vivo* hypercholesterolemic model was used to assess the hypocholesterolemic effect of fermented milks in rats with the previously demonstrated cholesterol-lowering potential effect.[19,21]

Rats ingested fermented milks and nonfermented milk *ad libitum* for 7 weeks. In this period, body weight gain was increased; however, no changes were observed between the control (SD) and the hypercholesterolemic groups. In fact, the food intake was lower in the hypercholesterolemic group. Similar results were previously reported by the administration of diets with a high cholesterol or a high-fat high cholesterol diet.[28,29] These increases in food consumption with changes in body weight may be related to low food efficiency and homeostatic mechanisms that impact the appetite response and daily caloric intake. Moreover, the relationship of food intake and body weight also depends on the hedonic factor (palatability) and the cognitive factors.[30,31] In this study, the consumption of fermented milks apparently did not exert an effect on either food consumption or body weight gain, suggesting that fermented milks did not regulate appetite. In fact, results suggested that dietary cholesterol intake was similar in rats with a high-cholesterol diet, since food intake was similar.

Using a high-cholesterol diet, hypercholesterolemia was induced for 7 weeks; in fact, blood sampling at week three indicated that total cholesterol was significantly increased. The results of induction were consistent with previously reported studies, indicating that a hypercholesterolemic diet (1% cholesterol) increases serum cholesterol levels to above 60%.[32,33] Besides, non-HDL-C was remarkably increased, as well as at the same time, HDL-C decreased, indicating an alteration of lipoprotein metabolism by cellular and molecular mechanisms that include the modulation of genes related to

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lipid metabolism such as the sterol regulatory element-binding proteins (SREBPs).[34] Furthermore, the low levels of HDL-C are related to alterations of lecithin cholesterol acyltransferase (LCAT) activity, since this enzyme catalyzes free cholesterol esterification for efflux of cholesterol by HDL; in this sense, LCAT deficiency results from low HDL-C levels.[35] Plasma triglyceride levels by the high-cholesterol diet were not affected, and this can be explained by the high activity of lipoprotein lipase. This enzyme increases its activity in a hypercholesterolemic state, and consequently, triglycerides from chylomicron and VLDL-C are hydrolyzed to fatty acids preventing hypertriglyceridemia.[28] On the other hand, in this study, the alteration levels of cholesterol positively correlated with an increase of cardiovascular risk indexes. This association has been well established in coronary heart diseases in several large population cohorts.[36,37]

The consumption of fermented milks prepared with *L. lactis*, specifically with FM-572 and FM-600, showed the capacity to attenuate hypercholesterolemia in rats by decreasing total cholesterol and non-HDL-C in comparison with NFM. Moreover, FM-600 improved HDL-C levels in comparison with all other milks. Is noteworthy to mention that HDL-C has antiatherosclerotic action mainly by promoting reverse cholesterol transport from peripherial tissues to the liver, avoiding the accumulation of cholesterol and decreasing the development of atherosclerotic lesions.[38,39] Fermented milks also exert a positive effect in the liver, preventing lipids, cholesterol, and triglyceride accumulation.

Cholesterol metabolism is mostly regulated by exogenous and endogenous pathways. The liver is the major organ for lipid metabolism which regulates the absorption, synthesis, or excretion of cholesterol and bile acids.[40] The increase of the hepatosomatic index observed in this study is related to an increase of lipid droplet deposition; in fact, in this study, the hepatosomatic index was significantly associated with triglyceride levels, suggesting a development of steatosis or NAFLD in the hypercholesterolemic groups. However, the consumption of fermented milk may exert a positive effect in NAFLD by decreasing lipid accumulation. Furthermore, the correlation analysis indicates that hepatic cholesterol was negatively associated with fecal cholesterol suggesting that lipid metabolism is closely associated with an increase of cholesterol excretion by the bile in the form of neutral sterols, bile acids, or cholesterol. This excretion is regulated by the ATP-binding cassette subfamily G members 5 and 8 (ABCG5/8) and Niemann-Pick C1 like 1 (NPC1L1) proteins.[41] Previous studies showed that casein-derived peptides are capable of regulating cholesterol excretion by the activation of ABCG5/8.[42] In fact, the present study indicates that total lipids were slightly more excreted by the consumption of FM-572. Nevertheless, the cholesterol and bile acid excretion were equal in all hypercholesterolemic groups. Also, no significant correlations were found between plasma cholesterol and fecal cholesterol or bile acids. This observation may indicate that lipid excretion is carried out in the form of free fatty acids and sterols, such as coprostanol. Interestingly, peptide fractions derived from these fermented milks showed the ability for inhibiting micellar solubility of cholesterol and bile acids.[21] Therefore, an increase in cholesterol and bile acids should be observed in feces. Nevertheless, these mechanisms cannot be discarded since the excretion of cholesterol and bile acids is not easily observed in models with a high-cholesterol high-bile acid diet.[43]

Additionally, it has been reported that gut microbiota play an important role in metabolizing cholesterol and bile acids. Specifically, groups of bacteria such as *Eubacterium coprostanoligenes* can produce coprostanol from cholesterol.[44] Besides, other gut microbes such as *Lactobacillus* can adsorb cholesterol in the cell surface of bacteria.[45] Therefore, it is possible that microbiota alter the composition of cholesterol and bile acids in the intestine. Moreover, the production of secondary bile acids by gut microbiota and subsequent absorption modulates the cholesterol metabolism through the Farnesoid X receptor (FXR) which leads to lower levels of cholesterol and triglycerides in the liver.[46] In this regard, fermented milks with specific LAB strains have also been demonstrated that modulate gut microbiota and decrease cholesterol in bloodstream. The modulation of gut microbiota and improvement of the lipid profile by fermented milks are usually associated with probiotic bacteria as the main effectors of the hypocholesterolemic effect.[12] In this study, the fermented milks were heat-treated at the end of fermentation to inactivate the bacterial activity. Thus, the beneficial effect of fermented milks used in this study may be due to the action of metabolites such as bioactive peptides. The *L. lactis* used in this study previously demonstrated high proteolytic activity, and thus, the hypocholesterolemic effect may involve the peptides released during fermentation.[21]

To the best of our knowledge, there is limited evidence suggesting that peptides derived from fermentation exert a hypocholesterolemic effect. In this regard, in a previous study, it was demonstrated that the low-molecular weight fraction (<10 kDa) from milk fermented with *Streptococcus thermophilus* TMC1543 exerted a hypocholesterolemic effect *in vivo*.[18] Nevertheless, the bioactive compounds were not identified. Peptides derived from casein and whey proteins hydrolyzed by specific proteases (e.g., trypsin, pepsin, or Neutrase) have widely been reported to have a hypocholesterolemic effect[42,47,48] but not by using LAB. These peptides can regulate cholesterol levels by multiple mechanisms including the inhibition of cholesterol absorption as well as the inhibition of cholesterol synthesis in enterocytes and hepatocytes by the inhibition of 3-Hydroxy-3-Methylglutaryl Coenzyme A (HMG-CoA) reductase. HMG-CoA plays an important regulation of cholesterol biosynthesis; thus, peptides capable of inhibiting this enzyme result in the reduction of LDL-C levels in the bloodstream. Nevertheless, molecular mechanisms that involve the activation of genes such as SREBP2 and CYP7A1 are also described for peptides.[49]

Furthermore, other compounds released during fermentation may also be responsible for the hypocholesterolemic effect. In this sense, exopolysaccharides (EPS) have exerted a cholesterol-lowering effect through their bile acid-binding capacity.[50] Also, EPS may play a role as a prebiotic and hence have potential to modulate gut microbiota.[51] Conversely, SCFA can modulate cholesterol metabolism by reducing total cholesterol and non-HDL-C.[52] Moreover, cellular components (e.g., cell wall) have been shown to decrease total cholesterol and LDL-C *in vivo*.[53] The regulation of cholesterol levels by these compounds (EPS and SCFA) includes the activation of SREBP2 and CYP7A which modulates the cholesterol synthesis and cholesterol uptake by hepatocytes thought the increasing of LDLR.[52,54,55] Moreover, SCFAs such as propionate and butyrate can inhibit cholesterol synthesis through the inhibition of HMG-CoA reductase.[55]

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3. GUT MICROBIOTA RESPONSE TO CONSUMPTION OF MILKS FERMENTED WITH SPECIFIC STRAINS OF *Lactococcus lactis* WITH POTENTIAL CARDIOPROTECTIVE EFFECT

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Gut Microbiota Response to Consumption of Milks Fermented with Specific Strains of Lactococcus lactis with Potential Cardioprotective Effect

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Abstract:	Gut microbiota is associated with diverse metabolism alterations, such as hypercholesterolemia. The strategies for promoting health benefits for gut microbiota include the consumption of fermented foods, such as fermented milks. Therefore, the present study evaluated the potential association between gut microbiota and the hypocholesterolemic effect of milks fermented with Lactococcus lactis NRRL B-571 (FM-571), NRRL B-572 (FM-572) and NRRL B-600 (FM-600). Fermented milks were administered to hypercholesterolemic Sprague-Dawley rats during seven weeks. At the end of the experimental period, fecal and colonic microbiota were characterized using 16S RNA gene sequencing. Also, the short chain fatty acids (SCFAs) were characterized in feces. Results showed that a high-cholesterol diet (HCD) altered the bacterial community in both fecal and mucosal samples. The consumption of fermented milks promoted changes in the richness and diversity of gut microbiota, although these were not significant ($p>0.05$). The levels of SCFAs were improved after fermented milks consumption. From all SCFAs, butyrate was negatively correlated with total cholesterol, LDL-C and positively correlated with HDL-C. Furthermore, Ruminococcaceae, Lactobacillaceae, Lachnospiraceae and Oscillospiraceae families, were negatively associated with total cholesterol, LDL-C and positively associated with HDL-C. In general, these families were increased in groups treated with fermented milks, specifically with FM-572. Thus, these results showed that the consumption of fermented milks could improve the abundance of beneficial bacteria families and thereby exert a positive effect on the regulation of cholesterol metabolism.	
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Abstract

Gut microbiota is associated with diverse metabolism alterations, such as hypercholesterolemia. The strategies for promoting health benefits for gut microbiota include the consumption of fermented foods, such as fermented milks. Therefore, the present study evaluated the potential association between gut microbiota and the hypocholesterolemic effect of milks fermented with *Lactococcus lacti*s NRRL B-571 (FM-571), NRRL B-572 (FM-572) and NRRL B-600 (FM-600). Fermented milks were administered to hypercholesterolemic Sprague-Dawley rats during seven weeks. At the end of the experimental period, fecal and colonic microbiota were characterized using 16S RNA gene sequencing. Also, the short chain fatty acids (SCFAs) were characterized in feces. Results showed that a high-cholesterol diet (HCD) altered the bacterial community in both fecal and mucosal samples. The consumption of fermented milks promoted changes in the richness and diversity of gut microbiota, although these were not significant ($p>0.05$). The levels of SCFAs were improved after fermented milks consumption. From all SCFAs, butyrate was negatively correlated with total cholesterol, LDL-C and positively correlated with HDL-C. Furthermore, *Ruminococcaceae*, *Lactobacillaceae*, *Lachnospiraceae* and *Oscillospiraceae* families, were negatively associated with total cholesterol, LDL-C and positively associated with HDL-C. In general, these families were increased in groups treated with fermented milks, specifically with FM-572. Thus, these results showed that the consumption of fermented milks could improve the abundance of beneficial bacteria families and thereby exert a positive effect on the regulation of cholesterol metabolism.

Keywords: hypercholesterolemia, short-chain fatty acids; lipid metabolism**;** hypocholesterolemic effect.

1. Introduction

Cardiovascular diseases (CVDs) are the major cause of mortality worldwide. There are several risk factors associated with the development of CVD such as hypertension, obesity and dyslipidemia (James et al., 2019) In recent years, the evidence suggests the interplay between gut microbiota and the development of CVDs (Tang et al., 2017). Gut microbiota comprises around 100 trillion microorganisms, particularly bacteria, dominated by *Firmicutes* and *Bacteroidetes* phyla. Moreover, this community also includes viruses, archaea, protozoa and fungi (Rinninella et al., 2019). A healthy, balanced microbiota is important for the maintenance of homeostasis through their metabolic and immunological functions. However, the alteration of structure and function of the microbiota affects various metabolic pathways that facilitates the development of several diseases such as CVDs (Shi, 2019). The underlying mechanism has been associated with the alteration of permeability and the dysregulated production of gut-derived metabolites such as lipopolysaccharide (LPS), secondary bile acids (SBA), trimethylamine N-oxide (TMAO), uremic toxins and SCFAs (acetate, propionate and butyrate) (James et al., 2019).

Given the important function of the microbiota in health, its modulation is an emerging potential therapeutic target to prevent or decrease the risk factors associated with the development of CVDs. Since diet plays an important function in the composition of microbiota, dietary interventions with specific foods or food components (*e.g.* fiber, oligosaccharides, proteins and peptides) have exerted positive effects (Leeming et al., 2019). Fermented foods such as kefir, kimchi, kombucha and milks fermented with lactic acid bacteria (LAB), have been associated with cardioprotective properties (*e.g.* antihypertensive, hypocholesterolemic and antiobesity) (Hadjimbei et al., 2022; Melini et al., 2019). In fact, fermented milks with specific strains of *Lactococcus lactis* demonstrated cardioprotective properties such as antihypertensive and antithrombotic activities (Beltrán-Barrientos et al., 2018; Rendón-Rosales et al., 2019; Rodríguez-Figueroa et al., 2013). The reduction of cholesterol levels is one of the beneficial effects of fermented milks (Kawase et al., 2001; Ali, 2016). In this respect, fermented milks with *Lactococcus lactis* NRRL B-50571, NRRL B-50572 and NRRL B-50600 showed capacity for reduce total cholesterol and non-HDL cholesterol in plasma. Also, these fermented milks reduce lipid levels in liver (Rendón-Rosales et al., 2023). However, the underlaying mechanisms were no investigated. Previously report indicate that the hypocholesterolemic effect was associate with the participation of peptide fractions released during the fermentation process (Rendón-Rosales et al., 2019). Nevertheless, it was also hypothesized that gut microbiota modulation by the consumption of fermented milks can also be implicated in the regulation of cholesterol levels by SCFA (acetate, propionate and butyrate) production and the increase of beneficial bacteria. To date, it still unknow if the hypocholesterolemic effect of these fermented milks with *Lactococcus lactis* may also related to gut microbiota. Therefore, the aim of this study was to characterize the structure of fecal and mucosal microbiota after consumption of milks fermented with *Lactococcus lactis* NRRL B-50571 (FM-571), NRRL B-50572 (FM-572) or NRRL B-50600 (FM-600) with potential cardioprotective effect in an hypercholesterolemic rat model.

2. Materials and Methods

2.1.Preparation of Fermented Milks

The fermentation of milk with *L. lactis* was performed following a procedure described by previous reports (Rendón-Rosales et al., 2023). Briefly, *Lactococcus* (*L.*) *lactis* NRRL B-50571, NRRL B-50572 and NRRL B-50600 were cultured in M17 broth supplemented with dextrose or lactose solution. Fresh cultures were inoculated (3% v/v) in heat-treated (110 °C/10 min) reconstituted (10% w/v) nonfat milk and incubated for 12 or 24 h. The resulting milk pre-inoculums were inoculated again in heat treated (80 °C/30 min) milk and incubated for 48 h at 30 °C to obtain the fermented milks. At the end of incubation, the fermentation process was stopped by heat treatment (75 °C/15 min) and subsequent cooling (4 °C). Fermented milks were stored at -20 °C until used in the *in vivo* study.

2.2. Animals and Experimental Design

Animal experiments were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals and the experimental protocol was approved by the Bioethics Committee of the Research Center for Food and Development (Spanish acronym, CIAD), Hermosillo, Sonora, México (CE/017/2019). Male Sprague-Dawley rats (six-week-old) were obtained from the University of Sonora (Hermosillo, Sonora, Mexico). Prior to the experimental period, the rats were maintained with a laboratory rodent diet (Labdiet 5001) and purified water during three weeks for acclimation in controlled conditions (20-22 °C, 50 ± 10% relative humidity and 12 h light-dark cycles). After this period, rats were randomized and divided into six groups (n = 6). Finally, groups received the diet and treatments (**Table 1**) *ad libitum* during seven weeks.

Table 1. Diets and treatments administered to Sprague-Dawley rats during seven weeks.

No.	Diets	Treatment
1	Standard diet	Water
2	High-cholesterol diet (HCD)	Water
3	HCD	Non-fermented milk (NFM)
4	HCD	Fermented milk with NRRL B-50571 (FM-571)
5	HCD	Fermented milk with NRRL B-50572 (FM-572)
6	HCD	Fermented milk with NRRL B-50600 (FM-600)

High-cholesterol diet (HCD) was formulated with 98.75% standard diet, 1% cholesterol and 0.25% cholic acid. FM: fermented milk.

2.3. Sample Collection for Microbiota and Lipid Analysis

Feces were freshly collected in the last three days before euthanasia of animals. Feces were mixed and immediately store at -80 °C in sterile conical tubes for further procedures. After the experimental period, the rats were euthanized by anesthesia administration (pentobarbital) by

intraperitoneal injection (75 mg/kg of body weight). Blood samples were obtained by intracardiac puncture and collected in heparinized tubes for further plasma collection (2500 *xg*, 15 min, 4°C). Plasma was used to determine total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG) and bile acids (BAs) with commercially available kits (Cell Biolabs, San Diego, CA). The colon tissue section was removed and microbiota from colonic mucosal was collected by scraping and kept in sterile conical tubes. All samples were immediately stored at -80 °C until bacterial genomic DNA extraction. For 16S RNA gene sequencing, either colonic mucosal or feces from two rats were pooled in equal amounts (100 mg).

2.4. Total DNA Preparation

Genomic DNA was extracted from feces and colonic mucosal scrapings using the QIAamp DNA stool minikit (Qiagen, Hilden, Germany) according to manufacturer's instructions with some modifications that include the use of Tris-HCl (10 mM, pH 8.5) for DNA elution in the final step. Quality and purity of DNA was analyzed by gel electrophoresis (1% agarose gel) and spectrophotometrically using Nanodrop 2000c (Thermo Fisher Scientific, Waltham, MA). Total DNA concentration was quantified using the Qubit dsDNA HS Assay Kit (Invitrogen, Carlsbad, CA) with a Qubit 4.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA).

2.5. Library Preparation and 16S rRNA Gene Sequencing for Fecal and Mucosal Microbiota Analysis

To investigate the bacterial community structure, the V3-V4 region of the 16s rRNA gene was amplified from bacterial DNA according to "16S-metagenomic sequencing library preparation guide" protocol. The first PCR amplification was performed using primers from V3-V4 region of the 16S rRNA gene that consists of forward Primer 5´-CCTACGGGNGGCWGCAG-3´ with the forward overhang 5´-TCGTCGGCAGC GTCAGATGTGTATAAGAGACAG-3´ and 16S Amplicon PCR reverse Primer 5´-GACTACHVGGGTATCTAATCC-3´ with the reverse overhang 5´-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG. Samples were amplified in reaction volumes of 12.5 μL containing 12.5 ng of total DNA and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA). PCR was performed with the following program: initial denaturation 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min. At the end of each PCR step, the products were purified using AMPure XP reagent (Beckman Coulter, Brea, CA). For library construction, purified DNA was used as second PCR template using primers from Nextera XT index kit v2 Set A (Illumina, San Diego, CA) that contain adapters and dual-index barcodes (index 1(i7) and index 2(i5)) (Illumina, San Diego, CA) complementary to the amplicon target. The second PCR program consisted of an initial denaturation at 95 °C for 3 min, followed by 8 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and extension at 72 °C for 5 min. The resulting libraries were purified using AMPure XP reagent and assessed for quality by gel electrophoresis using a 2% agarose gel. Libraries were quantified by Qubit dsDNA HS Assay Kit and normalized to a concentration of 4 nM. The library pool was then denatured with NaOH (0.1 N), diluted to 1.4

pM and mixed with denatured PhiX (1.4 pM) to a final concentration of 1% (v/v). Libraries were loaded into a MiniSeq High Output Reagent Kit (300-cycles). Automated cluster generation and paired-end sequencing with dual reads (2x151) were performed according to the manufacturer's instructions.

2.6. Bioinformatic Analysis

Data analysis was performed using the fastq files obtained from Illumina sequencing. First, the quality reads were assessed using FastQC software v 0.11.9. Then, the reads in Fastq.gz format were analyzed using DADA2 software package pipeline, running in R software (v 3.5.3). In the first step, the analysis included the quality filtering [maxN = 0, maxEE = c (2,2), truncQ = 8, length (truncLen= 140)]. After quality evaluation, only forward sequences were used for further analysis. Forward sequences were dereplicated and subsequent amplicon sequence variants (ASVs) were inferred. Next, chimeras were removed, and taxonomy was assigned against SILVA v138.1 reference database. ASV count, taxonomy and metadata tables were generated and subsequently utilized for analysis with the phyloseq package (McMurdie & Holmes, 2013). The unidentified sequences (NA) and those assigned to eukaryotic organisms such as mitochondria were discarded and abundance filter was also applied, removing families with less than 10 counts. Next, sequencing was randomly sub-sampled with rarefaction. Microbial diversity and composition were performed using microbiome R package (v 1.0.2). Alpha diversity was determine using Gini Simpson and Shannon indexes. The beta diversity for similarities in microbial communities between treatments was performed using Bray-Curtis. Beta diversity distance matrices were visualized with Principal Coordinate Analysis (PCoA). Analysis of compositions of microbiomes with bias correction (ANCOM-BC), with ANCOMBC package v 1.4.0, was performed for identifying the differentially abundant taxa (Lin & Peddada, 2020). Venn diagrams were employed to illustrate the results of the core and specific microbiota in mucosa and feces. These diagrams were generated using the web tool https://jvenn.toulouse.inrae.fr/app/index.html.

2.7. Determination of SCFA in Feces by Gas Chromatography

SFCAs were extracted in fecal samples according to a previously published protocol with some modifications in the chromatographic conditions (García-Villalba et al., 2012). Freeze-dried feces (50 mg) were weighed and 1.5 mL of 0.5% phosphoric acid was added and mixed for 2 min. Fecal suspensions were centrifuged at 18,000 *xg* for 5 min at 4 °C. The supernatant (1 mL) was collected, and an equal volume of ethyl acetate was added. Next, samples were mixed for 2 min, then centrifuged at 18,000 *xg* for 5 min at 4°C. The organic phase was transferred to a glass insert into an autosampler vial and spiked with hexanoic acid at a final concentration of 1000 uM. A 5-point of calibration curve (200-2500 uM) was prepared with a mixture of acetic acid, propionic acid and butyric acid, and hexanoic acid as internal standard. SCFAs quantification was performed by gas chromatography (GC) with flame ionization detector (FID). The GC system consisted of an HP 6890 (Hewlett Packard, Wilmington, DE), equipped with an automatic liquid sampler. The SCFAs were separated in a BD-FFAP capillary column at a flow of 1 mL/min using helium as carrier gas. Samples were automatically injected in a split mode (5:1 split ratio). The oven temperature program

was set with an initial temperature of 90 °C, increased to 150 °C at the rate of 10 °C/min, then to 170 °C at the rate of 5 °C/min and held for 10 min at 200 °C. Identification of the SCFAs was based on the retention time of analytical standard of SCFAs.

2.8. Statistical Analysis

All statistical tests for microbiota analysis were performed using non-parametric test using R packages. Statistical analysis for alpha diversity was performed using Mann-Whitney-Wilcoxon test and Kruskal-Wallis test. Analysis of similarities (ANOSIM) was used for significance calculation of beta diversity-PCoA with 999 permutations with vegan package (v1.4.0). Correlation analysis was carried out by using the Spearman coefficient via corrplot package (v 0.92). The differential abundance was performed using ANCOM-BC following by False Discovery Rate (FDR) for p value correction (q value) where the SD group was set as the reference level group. SCFAs data was analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer for means comparison in GraphPad Prism version 9.51. (San Diego, CA). The significance for all statistical analysis was considered when p value was < 0.05 and asterisks in bar plots indicate the p value: $^{****}p<0.0001$, $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, ns: not significant. Tendency was defined when p value was in a range of 0.05 to 0.1.

3. Results

3.1. Effect of a Hypercholesterolemic Diet and Fermented Milks in Microbiota Biodiversity

To investigate the effect of hypocholesterolemic fermented milks with *L. lactis* on gut microbiota in hypercholesterolemic Sprague-Dawley rats, a 16s rRNA gene sequencing was performed to analyze the bacterial community response in colonic mucosal and fecal samples. The alpha diversity analysis detected a significant difference in Shannon ($p = 0.0004$) and Simpson ($p = 0.0027$) indexes exhibiting a higher richness and diversity in colonic mucosal that in feces microbiota as seen in **Figure 1A.** PCoA based in Bray-Curtis distance showed a remarkably clustering pattern between both samples (**Figure 1B**) indicating differences (ANOSIM analysis, $p=0.001$) in the composition of microbiota in both samples. To investigate the impact of fermented milks consumption on microbial diversity an independent analysis was performed for either mucosal or fecal samples for the different treatments. There were no significant differences ($p>0.05$) among the different treatments either both fecal and mucosal samples as shown for the alpha diversity based in Shannon index (**Figure 2 A and B**). However, the consumption of FM-572 showed a tendency to increase diversity ($p=0.10$) when compared with the HC control (**Figure 2A**) in fecal sample.

Changes in microbiota structure were determined by Bray-Curtis dissimilarity. The Bray-Curtis analysis showed a significant distance separation (ANOSIM analysis, $p=0.037$) between the different treatments in feces (**Figure 2C**). Specifically, there was cluster separation among the HC control and FM-571, FM-572, and control groups, suggesting that the microbiota after the

consumption of fermented milks was improved since it was closer to the control group. For mucosal microbiota, clustering was observed between the control and all hypercholesterolemic groups as reflected in PCoA (**Figure 2D).** However, the consumption of fermented milks did not exert changes in the colonic mucosal microbiota composition (p=0.072).

3.2. Effect of Hypercholesterolemic Diet and Fermented Milks on Gut Microbiota Composition at Phylum and Family Level

The effect of the hypercholesterolemic diet and milks fermented with *L. lactis* on the relative abundance at phylum and family level were also analyzed. The predominant phyla in each group in feces and mucosal samples are shown in **Figure 3AB**. *Bacteroidota* and *Firmicutes* were the dominant phyla comprising around 94% of total phyla in feces, whereas, in mucosa, *Bacteroidota*, *Firmicutes* and *Desulfobacterota* were the most abundant phyla with more than 80% of total phyla. Moreover, *Actinobacteriota* phyla was only detected in fecal microbiota.

The high cholesterol diet increases the abundance of *Bacteroidota* and decrease *Firmicutes* phylum in feces ($p < 0.05$). In mucosa, the relative abundance of both *Bacteroidota* and *Firmicutes* phylum was increased ($p < 0.05$) in hypercholesterolemic control compared to healthy control. Moreover, the abundance of *Campylobacterota* decreased after high cholesterol diet. Furthermore, NFM, FM-571 and FM-572 increased the abundance of *Firmicutes*, and FM-572 decreased the relative abundance of *Bacteroidota* phylum ($p < 0.05$) in fecal microbiota (**Supplemental Table 1**). Nevertheless, no significant ($p>0.05$) changes were observed by the administration of the different fermented milks at phylum level in mucosal microbiota. A *Firmicutes* to *Bacteroidota* ratio was also determine for both samples; however, no significant changes were detected ($p>0.05$) (**Figure 3C and D**). Nevertheless, the administration of FM-571 and FM-572 slightly increased *Firmicutes*/*Bacteroidota* ratio in mucosa (**Figure 3D**) compared with HC control (p=0.078).

At family level, 29 and 24 families were identified in fecal and mucosal samples; respectively (**Supplemental Figure 1**). All 24 families that were found in mucosa were also identified in feces, however, *Coriobacteriales Incertae Sedis*, *Eggerthellaceae*, *Monoglobaceae*, *Morganellaceae* and *Streptococcaceae* families were exclusive to fecal samples. **Figure 4** shows the relative abundance at family level in fecal and mucosal microbiota. In feces (**Figure 4A**), *Muribaculaceae*, *Prevotellaceae* and *Lachnospiraceae* were the most predominant families comprising around 70% of total families. Meanwhile in mucosal microbiota (**Figure 4B**), *Desulfovibrionaceae*, *Helicobacteraceae* and *Muribaculaceae* families were the representative families in mucosa with above 50% of total bacterial groups.
ANCOM-BC analysis was used for identifying the most differential abundant families among the different treatments with control group as a reference group level. The analysis revealed that in feces, *Peptococcaceae* family was increased in the hypercholesterolemic group. However, *Ruminococcaceae*, *Oscillospiraceae*, *Anaerovoracaceae* and *Rikenellaceae* were decreased in this group (**Supplemental Figure 2**). In mucosa, *Eubacterium coprostanoligenes* group,

Lachnospiraceae, *Bacteroidaceae*, *Erysipelotrichaceae*, *Tannerellaceae* families were significantly ($p<0.05$) more abundant in the hypercholesterolemic control compared to Standard diet group ($p<0.05$). whereas *Lactobacillaceae*, *Saccharimonadaceae* and *Christensenellaceae* were differentially less abundant in the HC control (**Supplemental Figure 3**).

The consumption of fermented milks modifies the relative abundance of specific taxa, and this effect was observed mainly in fecal that in mucosal microbiota. In fecal microbiota, the consumption of fermented milks significantly increased the relative abundance of four specific families. First, *Lachnospiraceae* was increased with the consumption of all milks ($p<0.05$). Furthermore, FM-572 increased up to sixteen-fold of relative abundance of *Lactobacillaceae*. Besides, the relative abundance of *Oscillospiraceae* and *Ruminococcaceae* families was increased in FM-572 group in up to two-fold ($p<0.05$) as seen in **Figure 4 C**. In mucosal microbiota, the consumption of fermented milks did not exert an effect on the relative abundance of the different families.

3.3. Effect of Fermented milks in Short Chain Fatty Acids Production and their Association with Microbiota Families

To explore the effect of fermented milks on the production of SCFAs (acetate, propionate and butyrate), concentrations were determined in fecal samples at the end of the experimental period (**Figure 5A**). Total SCFAs was decreased in HC control; however, the consumption of fermented milks increased total SCFAs, specifically with FM-572 and FM-600 compared with HC control and NFM. Acetate was higher ($p<0.05$) in -FM-572 and FM-600, compared with the HC control or NFM ($p<0.05$). Propionate was increased in all hypercholesterolemic groups, except for NFM group, and the most significant ($p<0.05$) increases were observed in FM-572 and FM-600. Butyrate was notably decreased ($p<0.05$) up to 2.5-fold in all hypercholesterolemic groups. Nevertheless, the consumption of all fermented milks, increased ($p<0.05$) the levels of butyrate in feces compared to HC or NFM. **Figure 5** shows the correlation matrix that indicates the potential association between SCFAs, and the most abundant families identified in fecal and mucosal microbiota. As shown in **Figure 5B and C**, acetate was positively ($p<0.05$) correlated with *Lactobacillaceae* and *Oscillospiraceae* in feces and mucosa, while *Prevotellaceae* only in mucosa. Propionate was positively ($p<0.05$) correlated with *Oscillospiraceae*, *Maribaculaceae*, *Bacteroidaceae* and *Desulfovibrionaceae*; whereas butyrate was positively ($p<0.05$) correlated with *Rumminococcaceae*, *Lactobacillaceae*, *Christensenellaceae*, *Eubacterium coprostanoligenes* group and *Oscillospiraceae*.

3.4. Potential Association of Gut Microbiota with Plasma Lipid Levels

Plasma lipid levels (**Supplemental Figure 4**) showed a significant hypocholesterolemic effect in rats treated with fermented milks; specifically, those administered with FM-572 and FM-600, reducing TC and LDL-C levels ($p<0.05$). The potential association between lipid variables (total cholesterol, LDL-C, TG, HDL-C, plasma bile acids and fecal cholesterol) and the most abundant taxa at family level in feces and mucosal samples was explored. As shown in **Figure 6**, TC, LDL-

C and TG were positively associated with *Helicobacteraceae* and *Muribaculaceae* ($p<0.05$) in both samples. However, *Oscillospiraceae* in mucosa (**Figure 6A**), *Lachnospiraceae, Rumminococcaceae, Anaerovoracaceae* and *Lactobacillaceae* in feces (**Figure 6B**) were negatively associated with TC. Moreover, *Eubacterium coprostanoligenes* and *Rumminococcaceae* were negatively associated with LDL-C ($p < 0.05$). Also, HDL-C levels were positively associated with *Rumminococcaceae*, *Lactobacillaceae* and *Lachnospiraceae* families in mucosa (**Figure 6A**), whereas in feces, HDL-C was positively correlated with *Acidaminococcaceae* and *Desulfovibrionaceae* (**Figure 6B**). Subsequently, SCFAs levels were correlated with lipid levels (**Figure 6C**). From all SCFAs only butyrate was negatively correlated with plasma levels of TC, LDL-C, TG ($p<0.05$) and positively associated with HDL-C ($p<0.05$). These observations collectively suggest that the increased production of butyrate in groups treated with fermented milks may play an important role in the regulation of cholesterol levels, and may be related to the increase of butyrate-producing bacteria.

4. Discussion

Over the last several decades, considerable efforts have been focused on the prevention of CVDs and their related risk factors such as hypercholesterolemia. Indeed, hypercholesterolemia is considered the main modifiable risk factor, and their current management by clinical guidelines comprise the consumption of specific functional foods (Catapano et al., 2020; Mach et al., 2020). Nevertheless, it is important to consider that lipid metabolism can also be altered by gut microbiota, and in a dysbiosis state, it can lead to a high risk of atherogenesis and the development of CVDs (Jonsson & Bäckhed, 2016). In this study, we characterized the bacterial community in hypercholesterolemic rats induced by a high-cholesterol diet and evaluated the effect of hypocholesterolemic fermented milks with specific *L. lactis* during seven weeks. At the end of the experimental period, important changes were observed in lipid profiles.

Concurrently, microbiota composition was altered by the consumption of a high-cholesterol diet establishing a hypercholesterolemic model with an altered microbiota. The alteration in gut composition was reflected in the decrease of beneficial families, such as *Lactobacillaceae* and *Ruminococcaceae*. These changes were consistent with previous reports, where the abundance of these families was low in people with high risk of stroke and primary hyperlipidemia (Gargari et al., 2018). It is noteworthy to mention that low abundance of these families is also associated with an inflammation state (Forbes et al., 2016), which therefore can increase the risk of the development of CVDs. Our results also reflected that the hypercholesterolemic diet influenced the growth of bacteria that metabolize bile acids and cholesterol such as *Bacteroidaceae* and *Eubacterium Coprostanoligenes* group. Interestingly, these taxa were correlated with plasma bile acids and cholesterol; respectively, suggesting their capacity to metabolize these compounds.

The consumption of fermented milks with *L. lactis* reduced cholesterol levels with an effect in the structure of the bacterial community, characterized by the presence of specific taxa at family level. *Peptostreptococcaceae, Lachnospiraceae, Eubacterium coprostanoligenes* and *Ruminococcaceae* were among the families that exhibited more abundance in groups treated with the different

fermented milks. These results may indicate that the consumption of fermented milks exert a positive effect in the growth of these families. Notably, these families were negatively correlated with cholesterol levels. Particularly, *Peptostreptococcaceae* was negatively correlated with LDL-C indicating their potential role in the regulation of cholesterol levels. Besides, previous reports have highlighted the role of *Peptostreptococcaceae* in maintaining the gut homeostasis and its presence in gut indicate a good health state (Fan et al., 2017).

As expected, the *Eubacterium coprostanoligenes* group was also negatively associated with LDL-C and fecal cholesterol. It is important to note that this family is capable of metabolizing cholesterol producing coprostanol, which is a weakly absorbable sterol in the intestine (Ren et al., 1996; Juste & Gérard, 2021). The correlations found in the present study may suggest that the *Eubacterium coprostanoligenes* group decrease dietary and endogenous cholesterol in the intestine, decreasing their intestinal absorption and a reduction of cholesterol in plasma. The *Eubacterium coprostanoligenes* group has been proposed as an adjuvant of hypercholesterolemia treatment; although, this effect in cholesterol levels has been only confirmed in animal models (Li et al., 1995). The present study also showed that the abundance of *Lactobacillaceae* and *Ruminococcaceae* families were affected by hypercholesterolemia; in fact, a negative correlation was also found with cholesterol levels and these taxa. This potential cholesterol regulation effect is likely SCFAs-dependent since these taxa were also positively associated with SCFAs.

The analysis of SCFAs revealed a remarkably decrease of butyrate levels in the hypercholesterolemic group indicating a microbial dysbiosis. A decreased in butyrate production has been widely reported in inflammatory bowel diseases (Forbes et al., 2016). Nonetheless, the consumption of fermented milks increases butyrate levels, as well as related butyrate-producing families such as *Ruminococcaceae*, *Lactobacillaceae* and *Lachnospiraceae*. In the present study, these families were positively correlated with butyrate, and butyrate was also negatively correlated with lipid levels (TC, TG and LDL-C), and positively correlated with HDL-C. Therefore, these potential associations indicate that butyrate plays a beneficial role in the modulation of lipid levels and this effect may be enhanced by the consumption of the fermented milks. Although butyrate is associated with the barrier function, due to their effect in permeability (Peng et al., 2009; Bach et al., 2018), recent evidence suggests that butyrate extend its function beyond the barrier function protection. In this sense, butyrate is associated with the modulation of lipid metabolism with the consequent reduction of cholesterol levels (Amiri et al., 2022, Zhang et al., 2022). Moreover, this effect depends on multiple mechanism pathways, including the modulation of gene expression of enzymes and proteins related to synthesis and catabolism of cholesterol. In fact, the regulation of sterol regulatory element-binding protein 2 (SREBP2) and low-density lipoprotein receptor (LDLR) is involved in this process. Butyrate via SREBP2 modulation can reduce the expression of 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR) which is the rate-limiting step enzyme in the synthesis of cholesterol (Friesen & Rodwell, 2004; Zhao et al., 2017). Also, butyrate can increase LDLR synthesis, which plays a role in the uptake of LDL-C by the hepatocytes, therefore regulating the levels of cholesterol in bloodstream (Yang et al., 2020).

Although in the present study, propionate was not significantly associated with lipid levels; this SCFA has been reported to have a cholesterol lowering effect. This discrepancy was observed despite the fact that propionate levels were improved after fermented milks consumption. Propionate has been reported with the capacity of inhibiting HMGCR and suppress the expression of Niemann-Pick C1-like 1(NPC1L1), resulting in a decrease biosynthesis and intestinal absorption; respectively (Haghikia et al., 2022).

Similarly, acetate has been shown to have a hypocholesterolemic effect by the catabolism of cholesterol through the increase of activity of cholesterol-7-α-hydroxylase enzyme reflecting in the synthesis of bile acids. However, no significant changes were found in bile acids levels in plasma by the consumption of fermented milks. It is noteworthy to highlight that butyrate also exerts an antihypertensive and anti-inflammatory effect, reduce oxidative stress, that collectively suggest a protective effect against atherosclerosis (Wang et al., 2017; Zhang et al., 2017; Amiri et al., 2022). In the present study, other bacterial taxa were negatively associated with cholesterol, such as *Anaerovoracaceae*, *Erysipelotrichaceae*, *Erysipelatoclostridiaceae*, *Christensenellaceae* and *Oscillospiraceae*. However, it is difficult to suggest the contribution of these taxa to lipid metabolism, since there are limited reports that demonstrate their involvement. In summary, these fermented milks exert an effect on the modulation of gut microbiota at mucosal and fecal level. The observed changes in specific families could contribute to a positive effect in the regulation of lipid levels. This effect may be related to the participation of SCFAs, specifically butyrate, raising the possibility that the hypocholesterolemic effect of fermented milks is mediated by butyrate-producing bacteria. Due the fact that the composition of fermented milks is complex and abundant in bioactive compounds, it is possible that the hypocholesterolemic effect is also mediated by other molecules such as bioactive peptides and exopolysaccharides, which may collectively exert a synergist effect. Further studies will be focused on the study of the underlying mechanisms associate with the hypocholesterolemic effect of the fermented milks with *L. lactis*

5. Conclusion

The results of this study suggest that the consumption of fermented milks with *L. lactis*, specifically with FM-572 has a potential role in the management of hypercholesterolemia, which involves the potential contribution of specific bacterial taxa. Of particular relevance to our findings was the association of *Lachnospiraceae*, *Ruminococcaceae* and *Lactobacillaceae* families with lipid levels and SCFAs, specifically with butyrate. Therefore, the hypocholesterolemic effect of fermented milks may be likely related to butyrate enhancement in feces and the increase of the abundance of butyrate-producing bacteria.

List of abbreviations

FM-571: Fermented milk with NRRL B-50571
FM-572: Fermented milk with NRRL B-50572
FM-600: Fermented milk with NRRL B-50600

NFM: Non-fermented milk
HCD: High-cholesterol diet
L. *Lactococcus*
SCFA: Short-chain fatty acids
TC: Total cholesterol
TG: Triglycerides
ANOSIM: Analysis of similarities
ANCOM-BC: Analysis of compositions of microbiomes with bias correction
ASVs: Amplicon sequence variants
PCoA: Principal Coordinate Analysis

Declarations

Ethics approval and consent to participate

The experimental protocol was approved by the Bioethics Committee of the Research Center for Food and Development (Spanish acronym, CIAD), Hermosillo, Sonora, México (CE/017/2019).

Consent for publication

Not applicable.

Availability of data and materials

The data support the findings in this study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conceptualization, B.V.-C. and M.A.R.-R.; methodology, M.A.R.-R.; M.C.E.-M and J.I.M.-R.; software, M.A.R.-R. and J.I.M.-R.; validation, M.A.M.-M. and H.S.G.; investigation, M.A.R.-R.; writing-original draft preparation, M.A.R.-R.; writing-review and editing, B.V.-C., L.M.B.-B., A.H.-M., and A.F.G.-C.; project administration B.V.-C. and A.F.G.-C.; funding acquisition, B.V.-C. All authors have read and agreed to the published version of the manuscript.

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Figure captions

Figure 1. Analysis of gut microbiota biodiversity comparing fecal and colonic mucosal samples **A**, Simpson, and Shannon indexes for alpha diversity in fecal and mucosal samples. **B**, PCoA of Beta diversity based in Bray-Curtis distances dissimilarity comparing fecal and mucosal samples. ANOSIM was used for statistical analysis in PCoA. For alpha diversity, the Mann-Whitney-Wilcoxon test was used for median comparison.

Figure 2. Analysis of gut microbiota biodiversity in fecal and mucosal samples. **A-B**, Shannon index for Alpha diversity in fecal and mucosal microbiota, respectively. **C-D**, PCoA of Beta diversity based in Bray-Curtis distances dissimilarity in feces and mucosa, respectively. ANOSIM was used for statistical analysis. n.s. no statistically significant differences ($p>0.05$) based in a non-parametric test comparison.

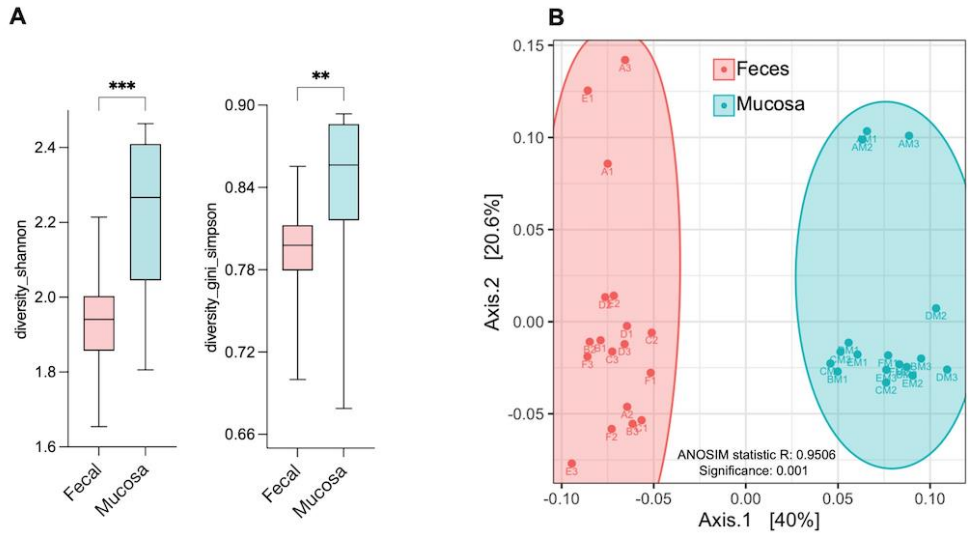
Figure 3. Gut microbiota composition at phylum level. Relative abundance of taxa at phylum level in **A**, in feces and **B**, in mucosa. Relative *Firmicutes*/*Bacteroidota* ratio for **C**, fecal and **D**, mucosal samples. Bars in C and D figures represent the median with range.

Figure 4. Gut microbiota composition at family level. Relative abundance of taxa at family level in **A**, fecal sample and **B,** mucosal sample. **C**, Effect of consumption of fermented milks with *L. lactis* level on the relative abundance (%) of specific families. Bars represent the median with range. Different letters indicate statistical differences ($p < 0.05$) between groups

Figure 5. **A** Concentration of short chain fatty acids (total SCFAs, acetate, propionate and butyrate) in feces of rats after fermented milks treatment. **B-C** Correlation Heatmap based on Spearman correlation coefficient between levels of SCFAs and specific families identified in mucosal and fecal samples. Asterisks indicate significative correlation (*, 0.05; **, 0.01; and ***, 0.001).

Figure 6. Correlation heatmap based on Spearman correlation coefficient between lipid levels (TC, LDL-C, HDL-C, TG, BAs, and fecal cholesterol) and the abundant taxa identified in **A)** Mucosa and **B)** Fecal microbiota. **C)** Correlation heatmap based on Pearson coefficient between lipid levels and SCFAs. Asterisks indicate significative correlation (*, 0.05; **, 0.01; and ***, 0.001).

Figure 1



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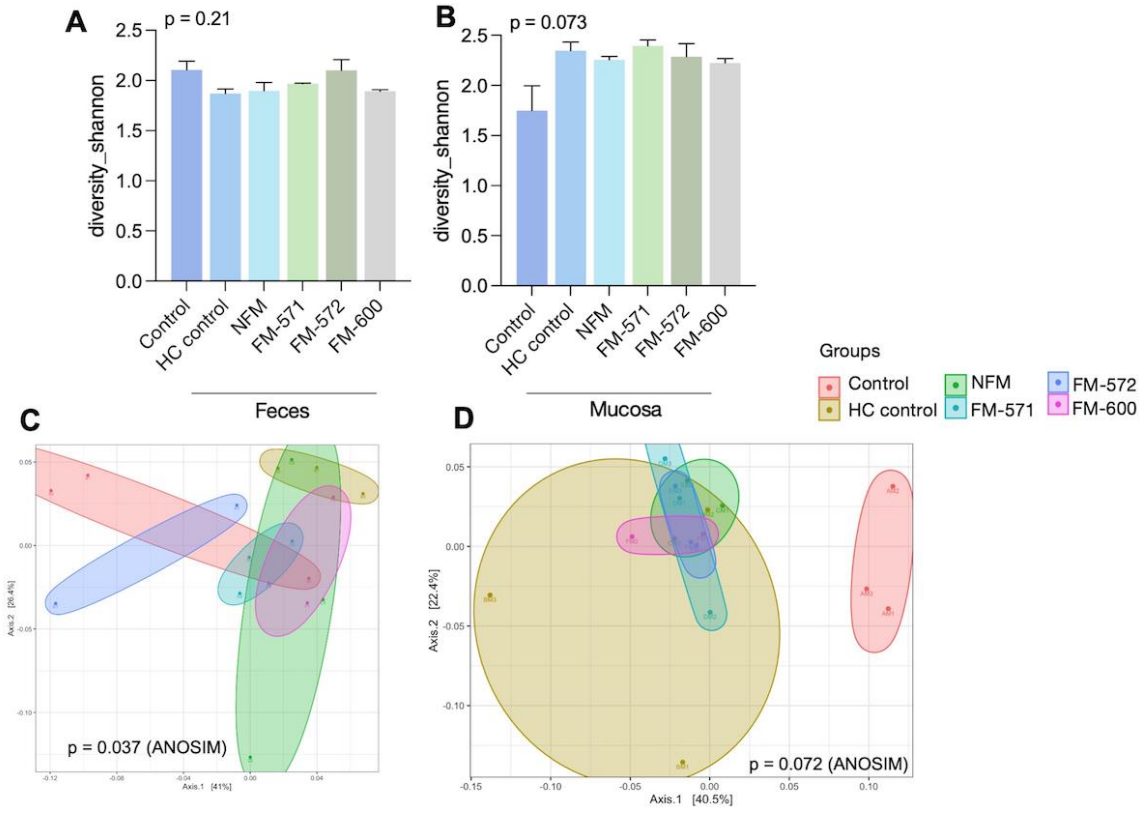

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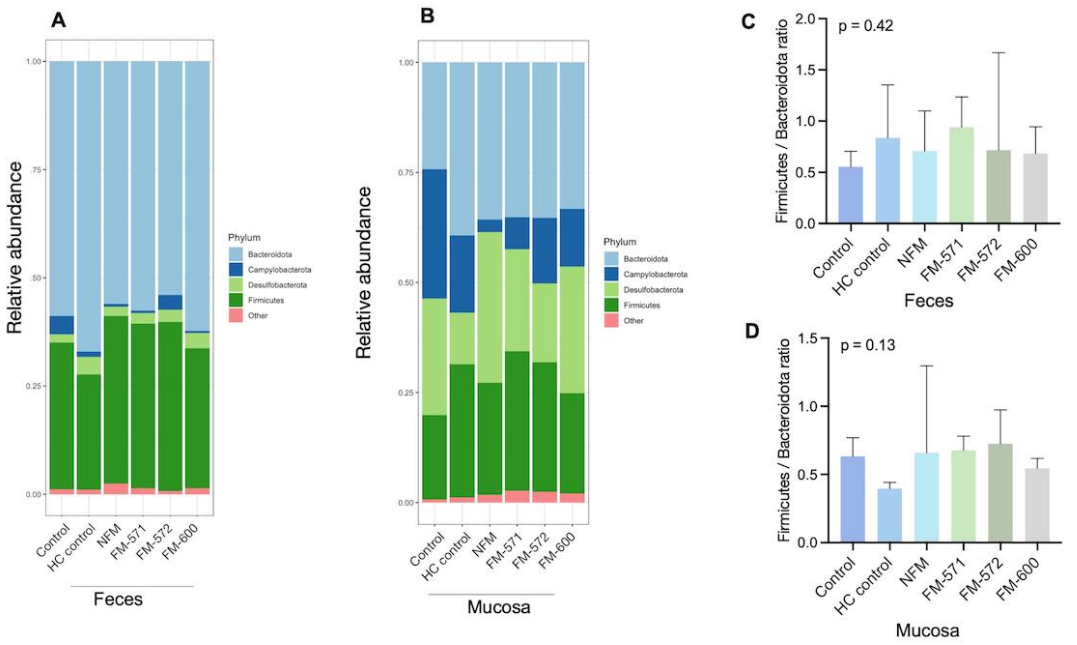

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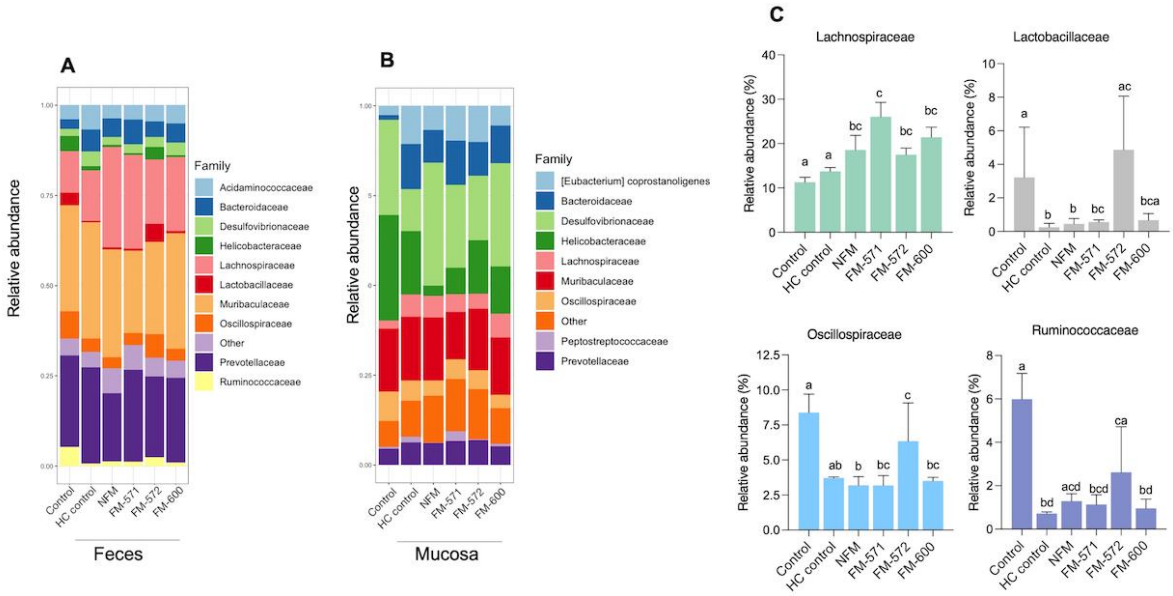
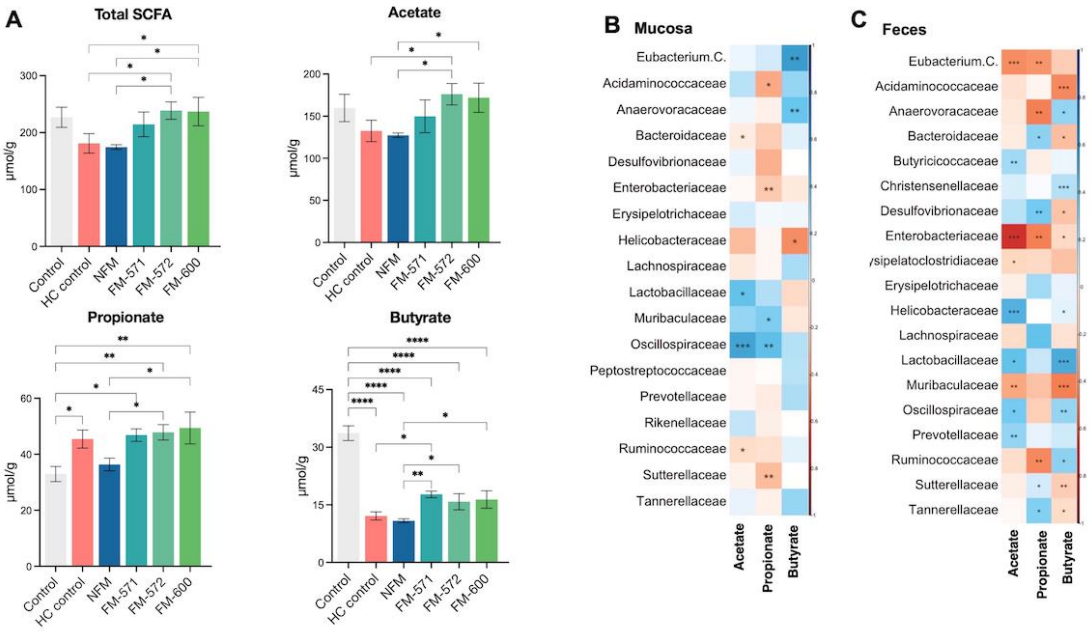


Figure 5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Total SCFA
Acetate
Propionate
Butyrate
µmol/g
Control
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NFM
FM-571
FM-572
FM-600
B Mucosa
C Feces
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Anaerovoracaceae
Bacteroidaceae
Desulfovibrionaceae
Enterobacteriaceae
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Lachnospiraceae
Lactobacillaceae
Muribaculaceae
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Rikenellaceae
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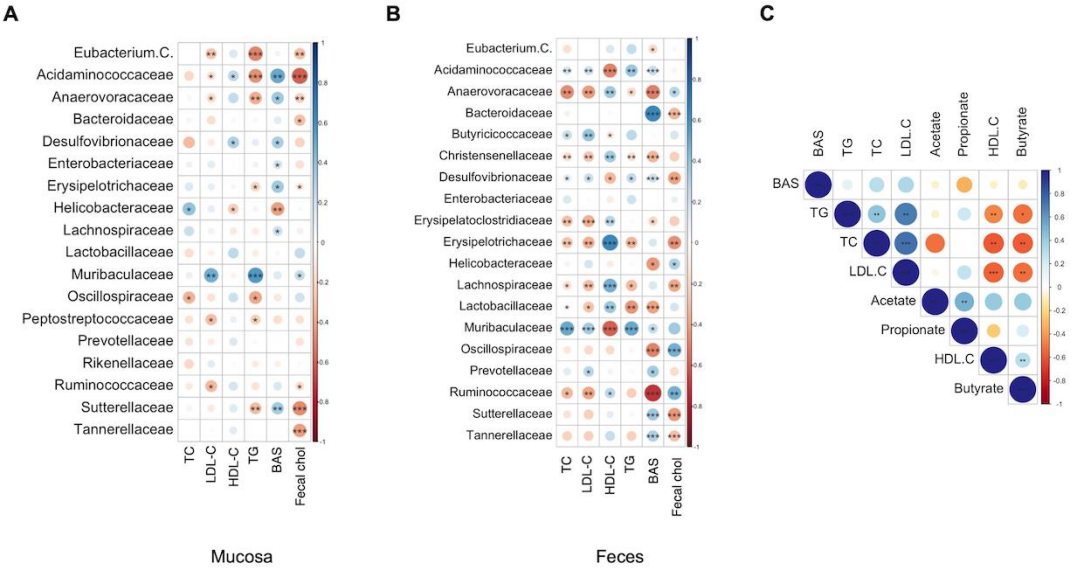
Figure 6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Sutterellaceae
Tannerellaceae
Butyricicoccaceae
Christensenellaceae
Erysipelatoclostridiaceae
TC
LDL-C
HDL-C
TG
BAS
Fecal chol
Acetate
Propionate
Butyrate
Mucosa
Feces

4. HYPOCHOLESTEROLEMIC ACTIVITY OF PEPTIDE FRACTIONS ISOLATED FROM MILKS FERMENTED WITH *Lactococcus lactis* NRRL B-50572 AND NRRL B-50600

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Manuscrito preparado para envío

Hypocholesterolemic Activity of Peptide Fractions Isolated from Milks Fermented with *Lactococcus lactis* NRRL B-50572 and NRRL B-50600

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ABSTRACT

The aim of this study was to evaluate the potential hypocholesterolemic effect of peptide fractions derived from milks fermented with *Lactococcus lactis* NRRL B-50572 (FM-572) and NRRL B-50600 (FM-600) subjected to simulated gastrointestinal digestion (SGD) and ex vivo peptide absorption. The inhibition of the micellar solubility of cholesterol (MSC) and the inhibition of the Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase were the mechanistic-based assays carried out in order to study hypocholesterolemic activity. Results showed that peptides significantly increase their abundance after SGD. Nevertheless, the abundance of peptides decreased after absorption ($p < 0.05$) in the ex vivo model. After SGD, the molecular weight of peptide fractions ranging from 0.29 to 0.62 kDa for FM-572 and FM-600 showed the best efficiency for inhibiting the MSC. Further, purification by RP-HPLC, showed that F3 and F4 from both FM were the active fractions for the inhibition of the MSC. Thereafter, all peptide fractions recovered in the basolateral compartment (absorbed peptides) were capable of inhibiting the HMG-CoA reductase enzyme. Nevertheless, absorbed peptides from FM-600 showed the best inhibition efficiency, despite the fact that peptides from FM-600 were less absorbed. Absorbed peptides from FM-600 were further fractionated by RP-HPLC. Results indicated that Fraction 1 was the fraction with the best efficiency for the inhibition of HMG-CoA reductase enzyme. The results suggest that the hypocholesterolemic effect of FM-572 and FM-600 involved the action of peptide fractions capable of inhibiting cholesterol micelles and cholesterol biosynthesis.

Keywords. Hypercholesterolemia, cholesterol biosynthesis, HMG-CoA reductase, cholesterol micelles, Bioaccessibility, bioavailability, cholesterol-lowering effect

INTRODUCTION

High blood lipids levels, such as present in hypercholesterolemia, is a risk factor involved in the development of cardiovascular diseases (CVD). Specifically, the elevated levels of atherogenic lipoproteins such as low-density lipoprotein cholesterol (LDL-C) promote atherosclerosis development. (Rooy & Pretorius, 2014, Prassad & Mishra, 2022). The management of blood lipids include multiple approaches that comprises pharmacological treatment and changes of lifestyle. It is well stablished that the consumption of healthy foods, functional foods and the adoption of healthy dietary patterns are an essential component in the management of hypercholesterolemia. In fact, the adoption of healthy habits reduces LDL-C in a range 10 to 15 %, and subsequent reduction of the cardiovascular risk. Besides, the consumption of nutraceuticals and functional foods are recommended by clinical guidelines as strategy for lipid-lowering treatment due their potential hypocholesterolemic effect. (Tokgözoğlu et al., 2020).

Functional foods are rich in bioactive compounds that exert beneficial health effects such as blood cholesterol regulation (Chen et al., 2018). In this respect, bioactive peptides have been reported with potential hypocholesterolemic effect that can be exerted by multiple mechanistic pathways that comprises the exogenous and endogenous (intracellular) cholesterol metabolisms (Boachie et al. 2018). In this respect, hypocholesterolemic peptides can inhibit the micellar solubility of cholesterol in the intestinal lumen, resulting in less absorbed cholesterol in the intestine (Boachie et al., 2018, Jiang et al., 2020). Conversely, peptides capable of crossing the intestinal barrier can inhibit the hydroxymethylglutaryl-Coenzyme A (HMG-CoA) reductase in the enterocyte as well in the liver, decreasing cholesterol biosynthesis (Silva et al., 2021). The inhibition of this enzyme depletes intracellular cholesterol, increasing the LDL receptor (LDLR) expression and therefore increasing LDL-C catabolism. This process results in lower levels of LDL-C in the bloodstream (Lin et al., 2015, Boachie et al., 2018).

The effectiveness of the hypocholesterolemic effect by peptides is achieved by multiple factors that involve the quality of proteins and method of proteolysis. In this sense, milk fermentation by lactic acid bacteria (LAB) has been demonstrated to release multiple bioactive peptides (*v.g.* antihypertensive, antioxidative and immunomodulatory) (Hafeez et al., 2014). However, the hypocholesterolemic effect of milk-fermented derived peptides is still unclear, despite the multiple systematic and metanalysis studies revealing the effect of fermented milks on cholesterol levels (St-Onge et al., 2000, Ziaei et al., 2021, Savaiano et al, 2021). A comparative study demonstrated that the low-molecular weight fraction (< 10 kDa) from milk fermented with *Streptococcus thermophilus* exerted hypocholesterolemic effect compared with complete fermented milk, whey, and casein fractions (Kawase et al., 2001). Furthermore, the administration of milk fermented with *Lactococcus lactis* NRRL B-50572 reduced LDL-C in normocholesterolemic hypertensive rats (Rodriguez-Figueroa et al., 2013).

Moreover, further studies, showed that the water-soluble fraction from fermented milks with *L. lactis* showed capacity for inhibiting the micellar solubility of cholesterol in a simulated gastrointestinal digestion model (Rendon-Rosales et al, 2019). Hence, these reports suggested that low-molecular weight molecules such as peptides could be involved in the hypocholesterolemic effect in the fermented dairy products. Thus, the aim of this study was to evaluate the hypocholesterolemic potential effect of isolated peptide fractions from milks fermented with *Lactococcus lactis* NRRL B-50572 and NRRL B-50600 by the inhibition of micellar solubility of cholesterol and HMG-CoA reductase in a simulated gastrointestinal digestion model and an *ex vivo* peptide absorption assay.

MATERIALS AND METHODS

Bacterial strains and culture

Lactococcus lactis NRRL B-50572 (L572) and NRRL B-50600 (L600) were obtained from the culture collection of the Dairy Laboratory at the Research Center for Food and Development, A.C. (Spanish acronym CIAD, A.C., Hermosillo, Sonora, México). *L. lactis* were cultured in M17 broth with 5 % v/v of dextrose or lactose solution (10% w/v) according to a previous report (Rendon-Rosales et al, 2019). Next, fresh cultures were individually inoculated (3% v/v) in heat-treated (110 °C/30 min) reconstituted nonfat milk (10 % v/v) and incubated at 30 °C during 12 h or 24 h for L572 and L600, respectively. For the preparation of fermented milks with L572 (FM-572) and L600 (FM-600), the pre-inoculums were individually inoculated (3 % v/v) in heat treated (80 °C/30 min) nonfat reconstituted milk (10 % w/v) and incubated during 48 h at 30 °C. Finally, heat treatment (75 °C/15 min), following by cooling (ice bath) was applied to fermented milks in order to stop fermentation.

Simulated Gastrointestinal Digestion

FM-572 and FM-600 were subjected to simulated gastrointestinal digestion (SGD) according to a previously reported model (Kopf-Bolanz et al., 2012). Briefly, 9 mL of FM-572 or FM-572, non-fermented milk (NFM), or water (digestion blank) were mixed with 12.5 mL of artificial saliva solution, and the mixture was incubated for 5 min (pH 6.5). Then, 25 mL of gastric solution was added, mixed, and incubated for 120 min (pH 2.0). Afterwards, 25 mL of pancreatic solution and 12.5 mL of bile solution were added to the mixture and incubated during 120 min (pH 6.8). All the process was maintained at 37 °C in constant agitation (300 rpm) in an orbital bath shaker. Finally, the digestion process was stopped with heat treatment (80°C/15 min). For the inhibition of micellar solubility of cholesterol assay (MSC), in the digestion process, the bile solution was not added, and in this phase the bile solution was replaced with an intestinal electrolytic solution.

Analysis and Isolation of Peptides by Size Exclusion Chromatography

FM-572 and FM-572 or NFM subjected to SGD were ultrafiltrated using a 10 kDa cutoff membrane, and the < 10 kDa fractions were freeze-dried. 100 µL of sample (100 mg of lyophilizate in 1 mL of mobile phase) was injected in a Protein Purification System (AKTA Pure). Separation was performed using a size exclusion column (Superdex peptide 10/300 GL) and saline phosphate buffer (100 mM, pH 7.2, with 0.15 M NaCl) as mobile phase with a flow rate of 0.6 mL/min. The peptide signals were detected at 280 nm during 60 min. All collected fractions were pooled in four chromatographic fractions (CF 1-4) and further lyophilized for the inhibition of MSC assay. The molecular weight distribution of peptides was estimated using aprotinin (6.51 kDa, vitamin B12 (1.35 kDa) and tyrosine (0.18 kDa) as standards. Additionally, the abundance of peptides in each CF (without freeze drying) were determined by RP-HPLC at 214 nm under the conditions described below.

Isolation of Peptides from Fermented Milks by RP-HPLC

The CF derived from SEC with the highest inhibition of MSC was selected for a second peptide purification step using reversed-phase HPLC system (series 1200) equipped with an analytical-scale fraction collector. Peptide separation was conducted using a C18 column (Agilent Zorbax Eclipse AAA, 4.6 x 150 mm, 3.5 µm particle size) in a gradient elution. The mobile phase A consisted of 0.04 % v/v trifluoroacetic acid (TFA) in water and mobile phase B consisted of 0.03% (v/v) TFA in acetonitrile. The following gradient program was carried out: 0-16 min, 0-60 % B; 16-17 min, 60-95 % B; 17-18 min, 95-100 % B; 18-18.5, 100-0 % B, and maintained for 8 min for baseline equilibration between injections. Flow rate was 0.60 mL/min and peptides were detected at 214 nm and the injection volume was set to 20 µL. Four chromatographic subfractions (CSF) were collected, freeze-dried, and finally resuspended in water for MSC evaluation.

Hypocholesterolemic Activity by the Inhibition of Micellar Solubility of Cholesterol by Isolated Peptide Fractions

Artificial micelles were prepared according to previous reports with minor modifications (Kirana et al., 2005). Lipids with final concentrations of 0.5 mM cholesterol, 2.4 mM phosphatidylcholine and 1 mM linoleic acid were solubilized and mixed in a chloroform-methanol mixture (2:1). The lipid mixture was dried under a nitrogen stream and the lipids were suspended in phosphate-buffered saline (15 mM, pH 7.4, 132 mM of NaCl) with 6.6. mM of sodium taurocholate. Next, the solution was sonicated for 20 min and incubated for 2 h at 37 °C. Thereafter, 25 mg of CF, 50 uL of CSF or 25 mg of cholestyramine as positive control were mixed with 0.5 mL of micellar solution. The mixture was sonicated once (2 min) and incubated for 2 h at 37 °C. The mixture was centrifuged at 10 000 xg during 10 min and the supernatant was recovered and filtrated through 0.22 µm syringe filters. The intermicellar content of cholesterol in the filtrates was determined using an enzymatic assay kit (Randox Laboratories). The percentage of inhibition was calculated according to the equation below (Kirana et al., 2005). Additionally, results were also expressed as efficiency of inhibition (IER), for this, protein content was determined using the DC protein assay

based in Lowry´s method.

$$\text{Inhibition of MSC (\%)} = [C_0 - C_S/C_0)] \times 100$$
$$\text{IER} = \text{inhibition of MSC (\%)} / \text{protein content (mg/mL)}$$

Where C_0 represents cholesterol content in supernatant without sample; C_s is the cholesterol content in the supernatant of micelles treated with the sample.

Ex vivo Peptide Absorption Assay by the Inverted Sac Method

Jejunum from rats was used for the peptide absorption assay by the inverted sac method reported by Dixit et al., 2012. A total of 20 male Wistar rats (271 g body weigh; 8 weeks old) were obtained from Bioinvert (Ciudad de México, México). Rats were kept with ad libitum intake of a standard diet (Pet Foods, Ciudad de México, México) and purified water in controlled conditions (22 °C ± 2, 40-60 % relative humidity). Prior to the absorption assay, rats were anesthetized with sodium pentobarbital (80 mg/kg body weight) by intraperitoneal injection, followed by cardiac puncture. Immediately, the abdomen was opened by midline incision and the intestine was carefully removed from mesenteric attachments. A jejune segment (16 cm) was excised and transferred to a conical tube containing cold Krebs medium. Subsequently, the jejunum segment was carefully everted and placed in a glass apparatus (**Supplemental figure 1**). The basolateral side was perfused with 15 mL of Krebs medium and the apparatus was transfer to a 100 mL beaker (simulating the basolateral side) containing 80 mL of digested FM-572, FM-600, NFM or the digestion blank. The temperature of the assembly system was maintained at 37 °C with aeration of a 95:5 air/CO_2 mixture with magnetic stirring at 55 rpm in the beaker. After 45 min, samples were collected from the basolateral side and immediately stored to -80°C. This study that involved animals was approved by and performed in accordance with the Bioethics Committee of CIAD (CE/017/2019).

Determination of Peptide Absorption Derived from Fermented Milks

For elimination of cellular debris and recovering of peptide fractions, samples were collected from the basolateral side and were ultrafiltrated using centrifugal filters (10 kDa, Amicon Ultra-0.5, Millipore, Billerica, MA, USA) at 10 000 xg, 30 min at 4 °C. Next, the ultrafiltrated samples were analyzed by an HPLC system (series 1260, Agilent technologies, Waldbronn, Germany), equipped with an autosampler injector and a diode array detector (DAD). Peptide separation, detection and sample injection volume were under the same conditions as described above. Integration of total peaks was performed in an OpenLab Chromatography Data System. The relative peptide absorption was calculated with the following equation (Xie et al., 2013).

$$BA = (AUC_A/AUC_T) \times 100,$$

Where AUC_A is the total area of the absorbed digested sample, AUC_T is the total area of the digested sample.

Hypocholesterolemic Activity by the HMG-CoA Reductase Inhibitory Activity

The HMG-CoA reductase inhibitory activity of samples were determined using the HMG-CoA reductase assay kit. The experiment was carried out at 37 °C following the manufacturer's instructions. Briefly, each reaction consisted in adding the reagents in the following order, samples (10 µL) were dissolved in a final volume of 184 µL in assay buffer, 4 µL NADPH solution, 12 µL Hydroxymethyl glutaryl Coenzyme A and 2 µL of the catalytic domain of HMG-CoAR enzyme. Subsequently, the components were mixed, and the absorbance (340 nm) was read during 10 min at 37 °C. The specific activity of the enzyme was defined as µmol of oxidized NADPH/min/protein (mg). The results were expressed as percentage of inhibition and efficiency inhibition ratio (% inhibition of HMG-CoA reductase / protein concentration)

Peptide Isolation of Absorbed Peptides by RP-HPLC

Absorbed digested samples with the highest HMG-CoAR was next subjected to peptide fractionation through RP-HPLC. Fractionation was carried out with the conditions described above using a HPLC system (series 1100, Agilent Technologies, Japan Ltd., Tokyo, Japan) equipped with an analytical-scale fraction collector module. Fraction collection was initiated from the moment the peptide signal appeared. A total of six chromatographic fractions were collected and lyophilized for HMG-CoA reductase inhibitory activity assay as described above.

Statistical Analysis

Data normality test was performed before statistical analysis. Data with normal distribution were analyzed by analysis of variance (ANOVA) or t-student for two independent samples. and mean comparison was carried out using Tukey-Kramer test. Results were expressed as mean ± SD for independent replicates. All statistical analyses were performed using GraphPad Prism version 9.0.0 for iOS, GraphPad Software, San Diego, California USA, www.graphpad.com.

RESULTS AND DISCUSSION

Molecular Weight Distribution and Abundance of Peptides Derived from Fermented Milks After Simulated Gastrointestinal Digestion

To investigate the hypocholesterolemic potential effect of peptides fractions derived from milks fermented with *L. lactis*, fermented milks were first subjected to a simulated gastrointestinal digestion process. Next, peptides were isolated from the peptide fraction (< 10 kDa) obtained by chromatography and finally, the peptide fractions were assessed for their hypocholesterolemic activity *in vitro*. It has been previously reported that the length of peptides might influence the hypocholesterolemic activity by the inhibition of micellar solubilization of cholesterol (Ashraf et al., 2020). Hence, in this study, the peptide fractions from digested FMs were first isolated by size exclusion chromatography (SEC). **Figure 1A** shows the total abundance of peptides (< 10 kDa)

from digested milks. The abundance of peptides was higher in FM-572 that in NFM ($p < 0.05$). However, not significant changes were observed between FM-600 and NFM ($p < 0.05$).

Figure 1B depicts the typical elution profile and the molecular weight distribution of peptides from digested milks by SEC chromatography. In general, peptides derived from all milks after gastrointestinal digestion showed an estimated size below 2.8 kDa, that comprises around 25 amino acid residues. Hence, from this profile, four chromatographic fractions were collected from all digested milks and the abundance of peptides in each fraction were determined by RP-HPLC. **Figure 1C** illustrates the abundance of peptides in each chromatographic fraction. In general, peptides molecular weight ranging from 1.3 to 2.8 kDa (CF1) were less abundant in all milks. In NFM, peptides below 0.29 kDa (CF4) were the most abundant in all fractions. Conversely, in FM-572, peptides that ranged from 0.29 to 0.62 (CF3) were the most predominant.

In FM-600, peptides with molecular weight ranging from 0.62 to 1.3 (CF2) and 0.29 to 0.69 (CF3) were the most abundant from all fractions in this milk. The size of peptides length obtained in this study was consistent with previous studies. Kopf-Bolanz et al. (2012) reported that after gastrointestinal digestion of milk the resulting peptides were no longer than 20 amino acids residues (< 3 kDa). In fact, *in vivo* data support that peptides with less than six amino acid residues are predominant after final digestion. However, the length of peptides after digestion can differ depending on the type of process applied to milk (Kopf-Bolanz et al., 2014). The fermentation process induced protein degradation by the proteolytic system of LAB and therefore the pattern of molecular weight distribution of peptides differs from those non-fermented milk products after gastrointestinal digestion (Zou et al., 2023, Zhang et al., 2023). It is noteworthy to mention that the type of LAB strain used also influences the abundance and the length of peptides obtained after digestion as shown in this study, since the proteolytic system can differ from each bacterial strain. Thus, peptides released after the digestion process can exert specifically their bioaccessibility and bioactivity (Zhang et al., 2023).

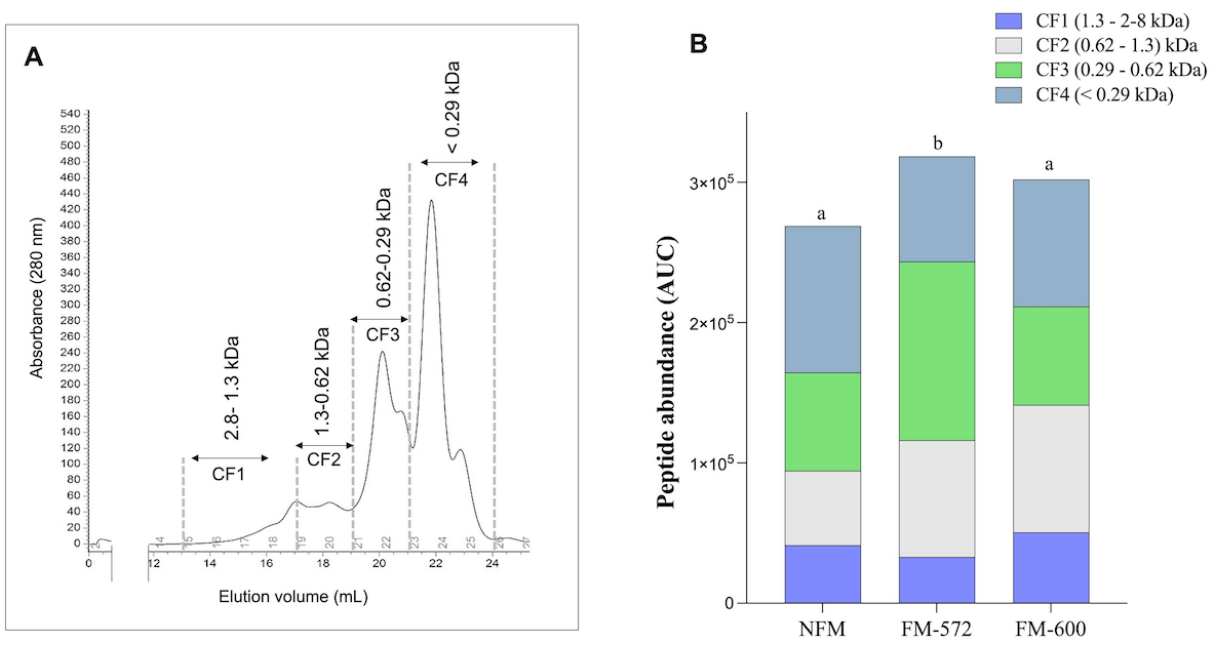

Figure 1. A) Typical elution profile of peptides by size exclusion chromatography that illustrates the collected fractions (CF1-CF4) and their estimated molecular weight. **B)**, Relative abundance of peptides and molecular weight distribution of collected fractions analyzed. Literals indicate statistical differences ($p < 0.05$) in total abundance of peptides expressed as area under the curve (AUC).

Inhibition of Micellar Solubility of Cholesterol by Isolated Peptide Fractions

The potential hypocholesterolemic effect of peptide fractions isolated from fermented milks was first evaluated through their capability to inhibit the micellar solubility of cholesterol (MSC). In a physiological process the formation of mixed micelles is essential for lipid absorption in the intestine. The micelles are composed mainly by bile salts, phospholipids, monoglycerides, fatty acids and dietary and biliary cholesterol (Woollett et al., 2006). In this regard, artificial micelles are formulated and used as a model for evaluating the potential hypocholesterolemic effect of bioactive compounds (Kinara et al., 2006). **Figure 2** shows the inhibition of MSC by the collected fractions isolated by SEC.

Results indicated that the percentages of inhibition (**Figure 2A**) were higher in collected fractions derived from FM-572 and FM-600 than from NFM and the inhibitory effect from all fractions ranged from 6.67 to 50.70 %. Nevertheless, CF3 from FM-572 showed the highest percentage of inhibition of MSC. The cholestyramine drug was also tested, and their percentage of inhibition of MSC was 79.84 %. The inhibitory efficiency ratio (IER) was determined based on the peptide concentration of each fraction. **Figure 2B** depicts the IER values for the inhibition of MSC. The inhibitory activity based on IER ranged from 17 to 198 (%/mg of protein). The fractions derived

from NFM showed the lower values of IER indicating the low efficiency to inhibit the formation of cholesterol micelles. Nonetheless, fractions from FMs exhibit higher inhibitory activity ($p < 0.05$) than fractions from NFM. Particularity, both CF3s from FM-572 and FM-600 showed the highest inhibitory activity ($p < 0.05$). These results indicate that peptides with estimated molecular weight in the range from 0.29 to 0.62 kDa of these FMs present the capacity to disrupt or inhibit micelles formation.

In previous studies, hypocholesterolemic peptides between this range of molecular weight were reported. Some examples of these peptides include IIAEK (MW 572.3 Da), HIRL (MW 537.3 Da), VPDPR (MW 582.3 Da), DPR (MW 386.1 Da) LPYP (MW 488.2 Da) and LPLPR (MW 594.38 Da) (Nagaoka et al., 2001, Takenaka et al., 2003, Takenaka et al., 2008, Yamauchi et al., 2003). Also, Zhong et al. (2007) found that hypocholesterolemic peptides based in the inhibition of MSC were in a range around 300-800 Da when using soy protein as a source of peptides. Likewise, Jian et al. (2020) found casein-derived peptides with inhibition of MSC with molecular weight in a range of 486.24- 659.35 Da. In addition to molecular weight, features such as hydrophobicity and isoelectric point are suggested as important properties of these peptides. However, the relationship between the structure of peptides and their capacity to inhibit cholesterol micelles is still unclear (Iwami et al., 1996, Beachie et al., 2018).

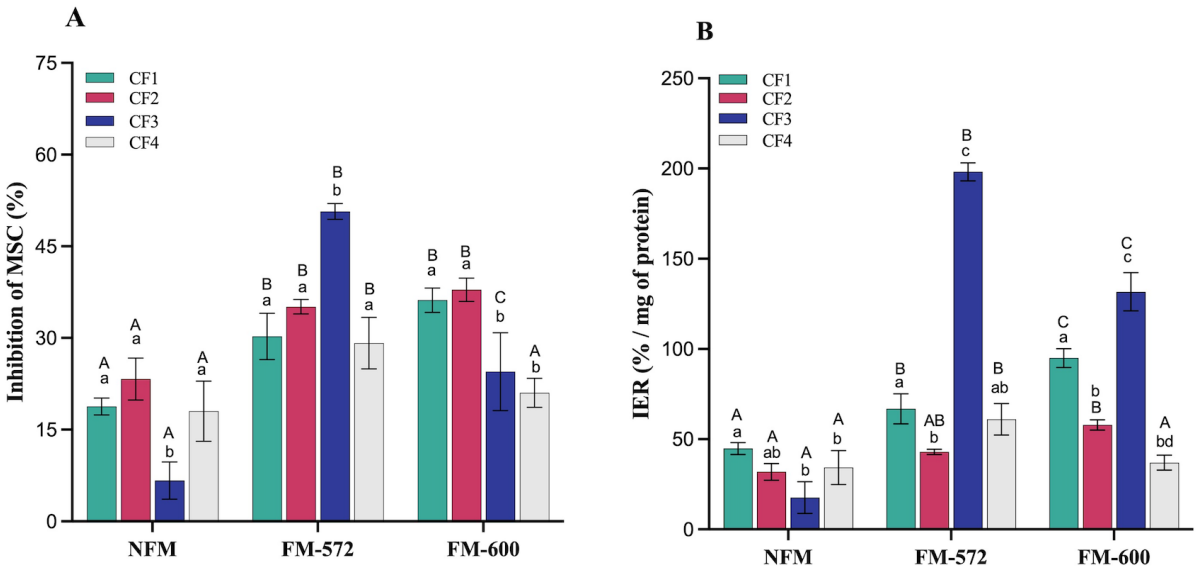

Figure 2. Inhibition of micellar solubility of cholesterol (MSC) by chromatographic fractions (CF1-4) obtained by size exclusion chromatography of FM-572, FM-600 and non-fermented milk (NFM). **A)** Percentage (%) of inhibition of MSC. **B)** Inhibitory efficiency ratio (IER) of MSC. The bars represent the mean ± SD. Lower case indicate statistical differences ($p < 0.05$) between fractions for the same milk. Uppercase letters indicate statistical differences between the different milks for the same CF.

Next, since CF3 from FM-572 and FM-600 exhibited higher efficiency of inhibition of MSC, these CF3 were selected for further purification by RP-HPLC. **Figure 3** illustrates the chromatographic peptide profile and its fractionation for the assessment of the inhibition of MSC. From these chromatographic profiles, four subfractions were collected and their MSC inhibitory activity are depict in **Table 1.** All subfractions exerted ability for inhibiting the MSC, and the percentages of inhibition ranged from 5.30 to 38.29 % for CF3-FM-572 and from 9.02 to 16.62 for CF3-FM-600. The IER was also determined and the subfractions with the highest efficiency for inhibiting the MSC was SF3 for CF3-FM-572, whereas for CF3-FM-600 were SF3 and SF4.

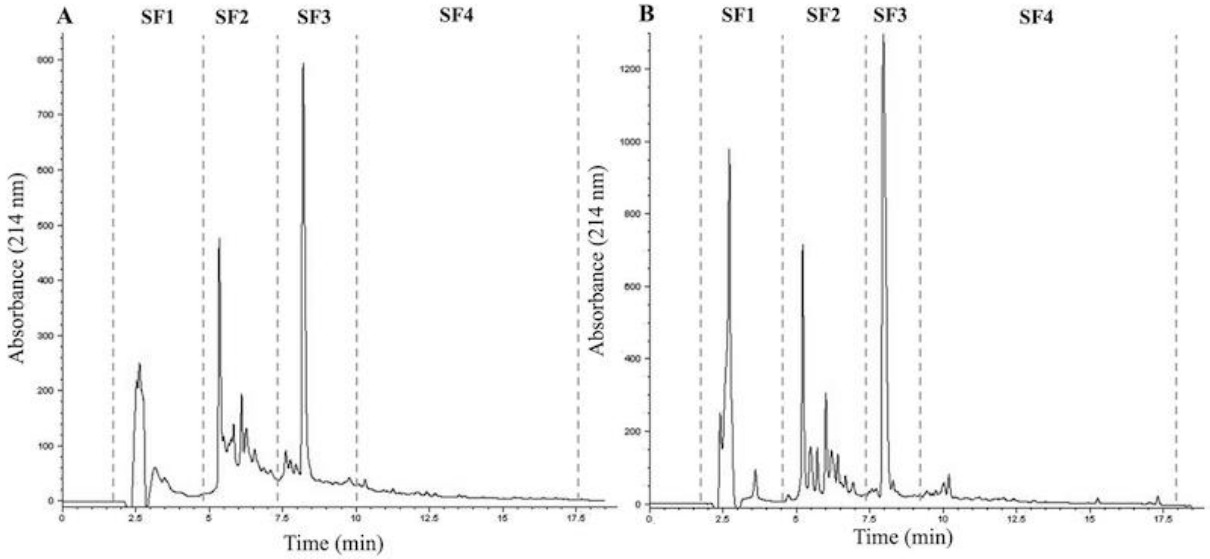

Figure 3. Chromatographic profile and fractionation of peptides from SEC-F3 derived from **A**) FM-572 and **B**) FM-600. SF: Chromatographic subfraction.

These active subfractions (SF3 and SF4) possibly contained hydrophobic peptides considering that the method of separation of peptides was by RP-HPLC. In this regard, these results are consistent with previous studies that indicated a relationship between hydrophobicity of peptides and the cholesterol lowering effect (Zhang et al.2012) since hydrophobic peptides are capable to interact with cholesterol. These peptide-cholesterol interaction limits the cholesterol incorporation into bile salts mixed micelles (Boachie et al., 2018, Acquah et al., 2018). Thus, the non absorbed cholesterol is excreted in feces or is metabolized in the colon (Zhang et al., 2013, Martínez et al., 2013). Also, peptides can decrease the micellar solubility of cholesterol by the interaction with bile salts (Imai et al., 2021). It has been described that Trp, Tyr, Phe, Leu and Val amino acid residues can bind to bile salts and therefore alter the process of solubilization of cholesterol. Example of bile acid binding peptides are PVRWKK, VAWWMY, KVWYMV (Takeshita et al., 2011).

The peptide-bile salt interaction decreases the critical micellar concentration reducing the efficacy of lipid absorption through the gastrointestinal tract. In fact, the low-molecular fraction of digested FM-600 showed in a previous study the capacity to bind to cholate and taurocholate bile salts

(Rendón-Rosales et al, 2019). Taurocholate, used in this study for micelles preparation, is reported to interact strongly with hydrophobic peptides as well as aromatics (Iwami et al., 1986, Matsuoka et al., 2007. The peptide-bile salt interaction is reported as a cholesterol-lowering mechanism due their effect in the enterohepatic circulation of bile salts, as result, the synthesis of bile salts is induced via 7-a-hydroxylation of cholesterol, reducing cholesterol in the bloodstream (Carrera-Alvarado et al., 2023). Hence, collectively our results indicate that the inhibition of the micellar solubility of cholesterol may be due to peptides with 3-6 six amino acids and composed by hydrophobic amino acids.

Table 1. Inhibition of MSC by chromatographic subtractions (SF1-SF4) from SEC fraction 3 (CF3) isolated from digested FM-572.

	CF3-572		**CF3-600**	
Subfraction	**Inhibition (%)**	**IER**	**Inhibition (%)**	**IER**
SF1	5.30 ± 2.69 ^{a}	57.74 ± 13.7 ^{a}	9.02 ± 2.25 ^{a}	124.14 ± 15.82 ^{a}
SF2	23.94 ± 8.36 ^{bc}	236.82 ± 27.02 ^{b}	9.56 ± 1.29 ^{a}	78.88 ± 12.34 ^{b}
SF3	38.29 ± 10.9 ^{b}	409.03 ± 90.5 ^{c}	16.62 ± 3.28 ^{b}	143.71 ± 22.81 ^{ac}
SF4	18.50 ± 3.39 ^{c}	319..51 ± 58.5 ^{ba}	11.83 ± 2.30 ^{ab}	154.23 ± 14.12 ^{c}

Ex vivo Intestinal Absorption of Peptides Derived from Fermented Milks

Bioactive peptides can decrease serum LDL-C by the inhibition of the enzyme HMG-CoA reductase localized intracellularly in the hepatocytes and enterocytes (Lin et al., 2015). In this regard, the bioaccessibility and bioavailability of peptides, specifically, the absorption of peptides, is important in order to exert the hypocholesterolemic effect (Silva et al. 2021). However, this important step is not considered in the studies of bioactivity. Therefore, in this study an *ex vivo* model was used for the assessment of the absorption of peptides for the evaluation of their capacity to inhibit the HMG-CoA reductase. First, the abundance of peptides during fermentation, gastrointestinal digestion, the absorption process and the non-absorbed peptides was determined. **Figure 4** depicts the peptide profile by chromatography, the abundance of peptides and the relative absorption of peptides. The chromatographic separation of peptides (**Figure 4A**) revealed a differential peptide profile depending on phase (fermentation, digestion, or absorption). Peptide peaks are increased after the fermentation process and more remarkably after the gastrointestinal digestion of fermented milks. This increased of peptides can be observed in **Figure 4B**. The abundance of peptides increased ($p < 0.05$) around thirteen-fold after gastrointestinal digestion.
Nevertheless, after the intestinal *ex vivo* absorption process, the signal of peptides (**Figure 4A**) as well the abundance of peptides (**Figure 4B**) markedly decreases ($p < 0.05$), and a large amount of peptides remain in the mucosal side (non-absorbable). Based in the abundance of absorbed peptides in relation to the abundance of peptides released in gastrointestinal digestion, the relative

absorption of peptides (%) was also determined. **Figure 4C** shows that peptides absorption ranged from 3.23 to 7.39 % and the peptides from digested NFM and FM-572 were the most absorbed compared with FM-600. Even though the abundance of peptides between NFM and FM-600 after gastrointestinal digestion was similar. The differences in the relative absorption of peptides possibly could be associated to the structure or physicochemical characteristics of these peptides. It has been widely reported that the absorption of peptides depends on the length of peptides and their amino acid composition, and therefore these features defined their absorption pathway (Shen & Matsui, 2017, Karás, 2019).

It is noteworthy to mention that the peptides released after gastrointestinal digestion of milks in the present study differ in the peptide length. The released of low-molecular weight peptides (<0.29, 0.29-0.62, **Figure 1**) was more abundant with NFM and FM-572 after digestion. This observation may suggest that di-tripeptides resulting from digestion and perhaps of brush border enzymes enhanced peptide absorption along with the intestinal barrier, resulting, therefore, in a high abundance of peptides in the basolateral compartment (Ozorio et al., 2020). Moreover, specific features also promote the interaction with other molecules such as proteins and lipids. Previously, peptide fractions from FM-600 showed capacity to bind bile salts, specifically with cholic acid and taurocholic acid (Rendon-Rosales et al., 2019). These interaction bile salt-peptide forms an unabsorbable complex that ends in the excretion of bile salts and peptides (Wang et al., 2015).

HMG-CoA reductase inhibitory activity by peptide fractions

HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis pathway and its activity regulated the *novo* cholesterol synthesis. In eukaryotes, HMG-CoA reductase are localized to the endoplasmic reticulum (ER) in the cell (Friesen & Rodwell, 2014). In hypercholesterolemia, this enzyme is a main therapeutic target and its inhibition results in the decrease of LDL-C in plasma (Suganya et al., 2017). In this respect, this study investigated the potential hypocholesterolemic effect of peptide fractions from fermented milks by the inhibition of HMG-CoA reductase. **Figure 5** shows the inhibitory activity of HMG-CoA reductase by peptide fractions (< 10 kDa) FM-572, FM-600 and NFM. Peptide fraction recovered after fermentation from FM-600 showed higher inhibitory (56%) activity compared with FM-572 and NFM ($p<0.05$). After gastrointestinal digestion, the percentage of inhibition decrease two-fold for all milks. Nevertheless, after *ex vivo* intestinal absorption only peptides absorbed from FM-600 maintain their inhibitory effect. The efficiency of inhibition (**Table 2**) reveals that peptide fractions from FM-600 showed the best inhibitory efficiency in all stages showed low values of IC_{50}

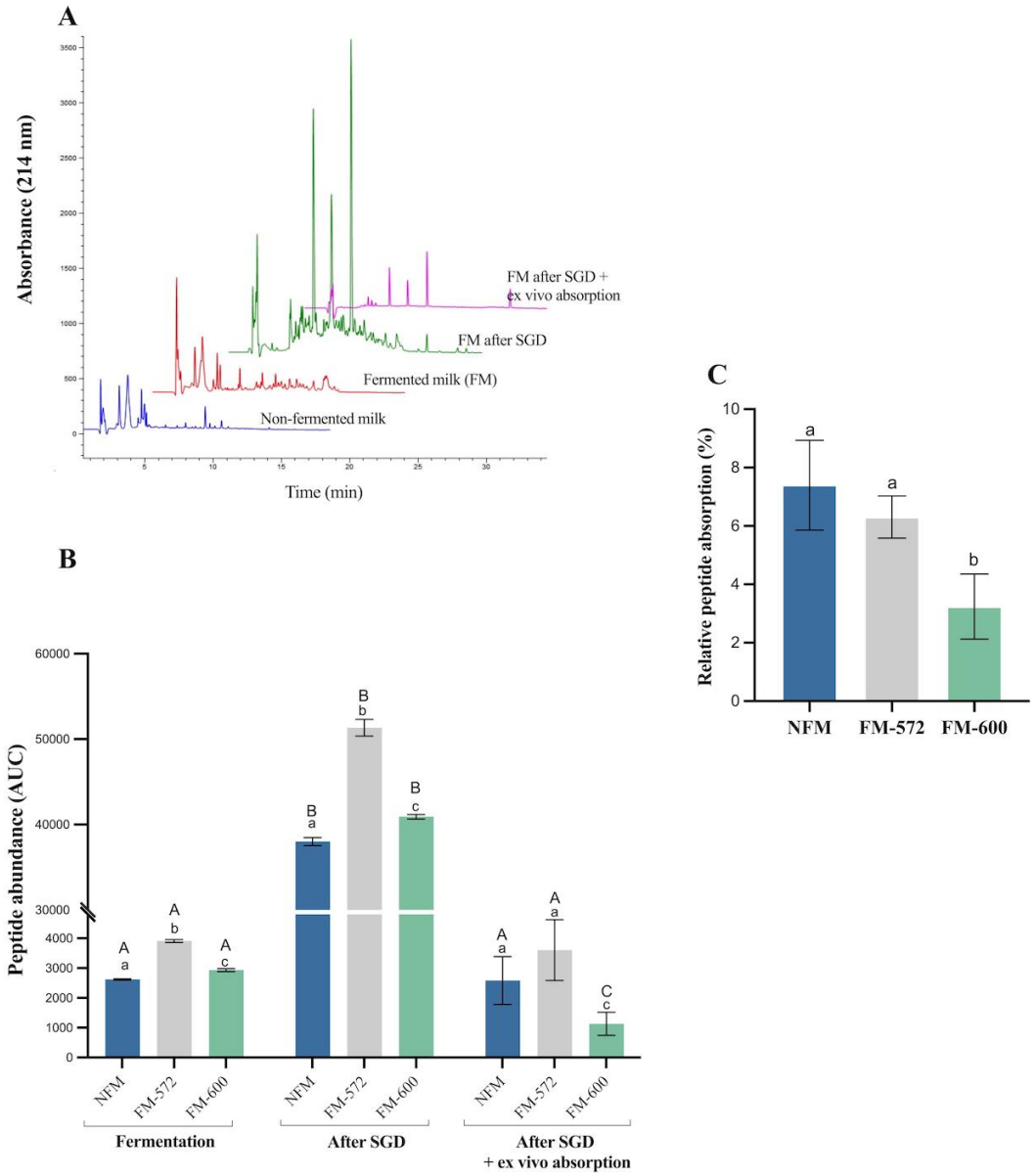

Figure 4. A) Representative chromatographic peptide profile of peptides of fermented milks (FM-572) obtained in the different stages. **B)** Peptide abundance expressed as area under curve (AUC) based in total area of peaks obtained by RP-HPLC of fermented milks and non-fermented milk in the different stages. **C)** Relative peptide absorption of peptides derived from fermented milks and non-fermented milk after gastrointestinal digestion. The different letter indicates statistical differences among milks in the same stage (lowercase), and the different stages for the same milk (uppercase).

Interestingly, despite the low absorption of peptides from FM-600, their inhibitory effect for HMG-CoA reductase was the highest. This observation suggests the differences in peptide structures among milks indicating the specify of structures that are released from fermentation and further hydrolysis in the gastrointestinal digestion, and by the brush border enzymes. Henceforth, the

bioaccessibility need to be considered for bioactivity analysis since it has been widely reported that bioactive peptides need to interact with a specific tissue or intracellularly. In this regard, peptides with HMG-CoA reductase need to be bioaccessible since their inhibitory action must be in the hepatocyte or enterocyte cells (Bechaux et al., 2019).

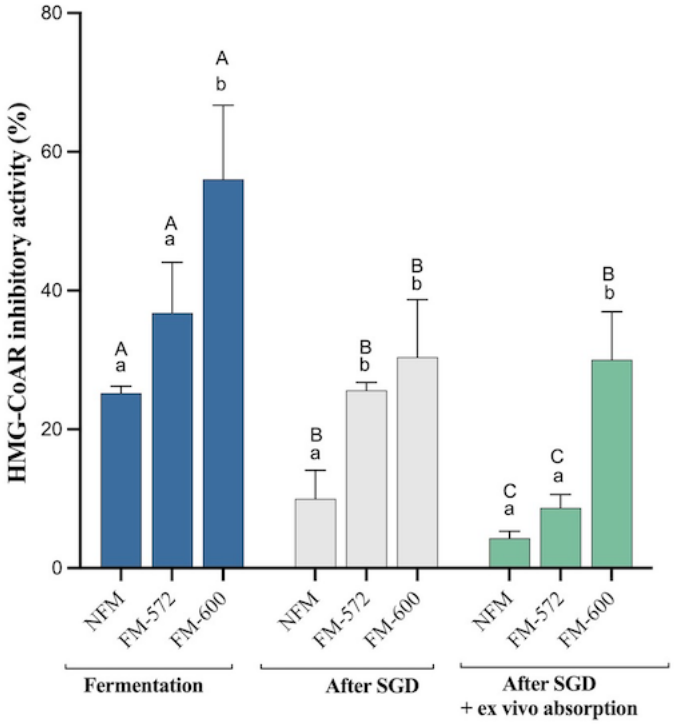

Figure 5. HMG-CoA reductase inhibitory activity (%) of peptide fractions (< 10 kDa) derived from fermented milks obtained at the different stages (fermentation, after gastrointestinal digestion and absorption process). The different letter indicates statistical differences among milks in the same stage (lowercase), and the different stages for the same milk (uppercase).

Table 2. HMG-CoA reductase inhibitory activity expressed as IC_{50} (mg prot/mL) by fermented milks

	Fermentation	**SGD**	**Absorption**
NFM	0.984 ± 0.04^{a}	2.12 ± 0.51^{a}	3.03 ± 0.68^{a}
FM-572	1.472 ± 0.28^{b}	1.33 ± 0.06^{b}	1.34 ± 0.25^{b}
FM-600	0.486 ± 0.08^{ca}	1.11 ± 0.31^{b}	0.284 ± 0.051^{c}

NFM: non-fermented milk; FM, fermented milk; SGD, simulated gastrointestinal digestion

The absorbed peptides from FM-600 were further fractionated by RP-HPLC and six chromatographic fractions were collected. **Figure 6A** illustrates the peptide elution and fractionation of peptides. The inhibitory activity of these chromatographic fractions is presented in **Figure 6B**. All fractions exert capacity for inhibiting the HMG-CoA reductase. From all fractions,

the firs eluted peptides below CF1 show the highest percentage of inhibition (>30%) and low IC_{50} value. Based on RP-HPLC separation, this fraction indicated that may contain hydrophilic peptides. However, it is necessary to characterize the sequences for establishing the structure-activity relationship. A similar study that involved intestinal *ex vivo* absorption of milk fermented with *Lactococcus. lactis* showed a wide variety of peptides in the basolateral compartment in a range from 0.207 to 1.173 kDa (Álvarez-Olguín et al., 2023). The released peptides showed a variety of structures that explain the mechanisms pathway of absorption and bioactivity. However, to the best of our knowledge, there are not reports of milk fermentation-derived peptides with HMG-CoA reductase inhibitory activity. Contrary to this, structures of peptides obtained from other sources (*v.g.* soy protein, amaranth) are available (Soares et al., 2015, Pak et al., 2005).

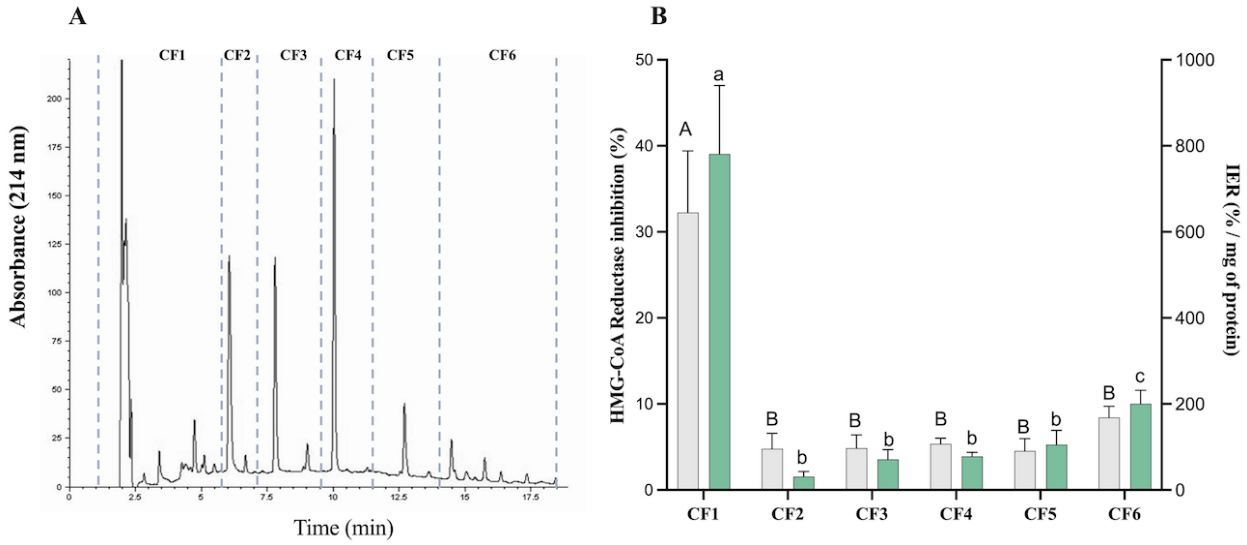

Figure 6. Chromatographic elution profile and fractionation (CF1-6) of absorbed peptide fraction derived from digested FM-600 (**A**), HMG-CoA reductase inhibitory activity expressed as percentage of inhibition (%) and inhibitory efficiency ratio (IER). Data represent the mean ± SD. Different literals indicate statistical differences between samples ($p < 0.05$) for HMG-CoA reductase inhibition (uppercase) and IER (lowercase).

Overall, structure-activity studies revealed that from those peptides present, at least four residues of amino acids (<1kDa) and the total hydrophobicity/hydrophilicity of peptides does not influence directly inhibition efficiency. Conversely, key features such as the peptide conformation and functional side such as the presence aromatic rings may have an influence inhibition efficiency. In this sense, tyrosine in the amino (N)-terminus or phenylalanine in the carboxyl(C)-terminus are important for the inhibitory activity of peptides with higher inhibitory activity (Pak et al., 2006, Silva et al., 2021). Also, Glutamic acid in this site enhances the inhibition by its recognition on the active site of the enzyme (Pak et al., 2022. In fact, the discovery of novel peptides is based in structural design of statins, (*v.g.* atorvastatin, rosuvastatin) which are competitive inhibitors of this drug. However, properties such as net charge, and hydropathicity are considered to enhance

bioavailability. In summary, the results indicated that fermented milks, specifically FM-572 and FM-600 can provide peptide fractions with capacity to disrupt micelle formation and decrease the enzymatic activity of HMG-CoA reductase.

CONCLUSION

The results of the present study demonstrated that milks fermented with *L. lactis* (FM-572 and FM-600) are a source of potentially hypocholesterolemic peptides that can exert their effect through the regulation of cholesterol metabolism by the exogenous and the endogenous pathways. Specifically, peptide fractions from FM-572 exert better capacity to disrupt cholesterol micelles. Conversely, FM-600 provides absorbable peptide fractions with potential capacity to inhibit cholesterol biosynthesis. Further studies are need to establish the structure-activity relationship and the *in vivo* cholesterol-lowering effect of these peptides.

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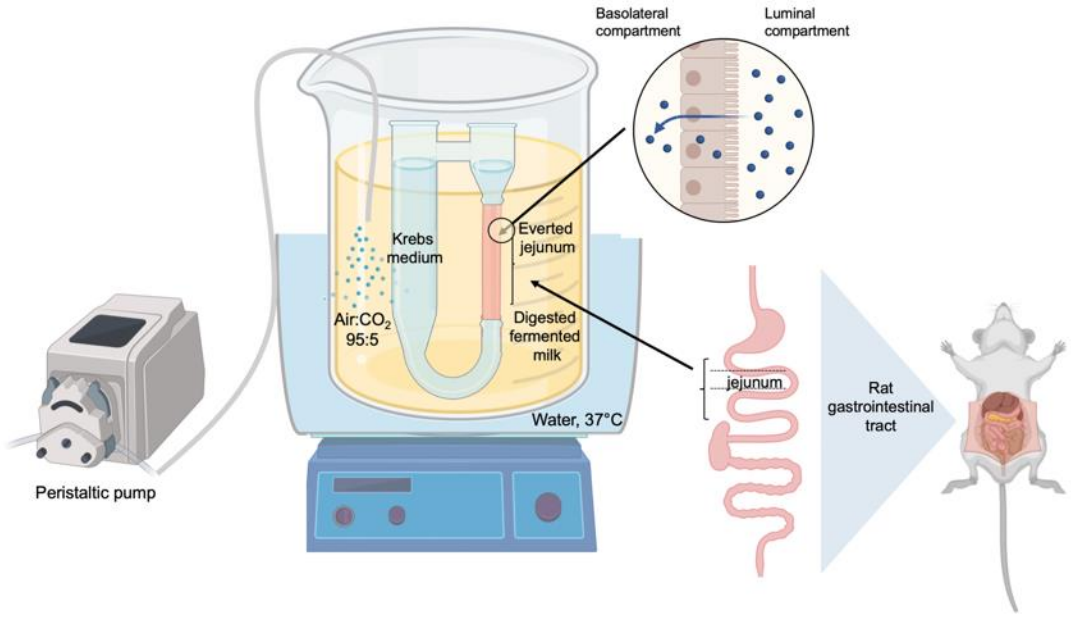
Supplementary Information

Supplementary Figure 1 (SF1)



SF1. Schematic representation of the *ex vivo* intestinal model for peptide absorption assay.

5. WATER-SOLUBLE PEPTIDE FRACTIONS FROM FERMENTED MILKS WITH *Lactococcus lactis* NRRL B-50572 AND NRRL B-50600 REGULATE BLOOD LIPIDS LEVELS IN AN HYPERCHOLESTEROLEMIC MURINE MODEL

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Manuscrito preparado para envío

Water-soluble peptide fractions from fermented milks with *Lactococcus lactis* NRRL B-50572 and NRRL B-50600 regulates blood lipid levels in an hypercholesterolemic murine model

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ABSTRACT

Previous studies reported that fermented milks with *Lactococcus lactis* exert hypocholesterolemic effect *in vivo*. However, the components that explain the effect is unclear. In vitro studies suggested that components with a low molecular weight (< 10kDa) such as peptide fractions may be implicated un the cholesterol-lowering effect. Nevertheless, the in vivo effect is unknown. Therefore, the aim of this study was to evaluate the *in vivo* hypolipidemic effect of water-soluble fractions (low molecular weight fraction < 10 kDa) derived from milks fermented with *Lactococcus lactis* NRRL B-50572 (WSF-572) or NRRL B-505600 (WSF-600). Sprague-Dawley rats were randomly assigned in four groups. The treatments for each group consisted of 1) Standard chow diet + purified water; 2) high-cholesterol diet (HCD) + purified water; 4) HCD + WSF-572; and 4) HCD + WSF-600. After five weeks of intragastric administration, lipid profile (total cholesterol, LDL-C/VLDL-C, HDL-C, triglycerides, and non-esterified fatty acids (NEFA) were determined in plasma. Also, the excretion of cholesterol, bile acids and NEFA were also determined in feces. After the administration perido, body weight gain was significantly lower ($p < 0.05$) in groups treated with both WSF. Furthermore, the administration of WSF exerted hypolipemic effect since the administration of both WSF decreased ($p < 0.05$) plasma total cholesterol (21.21-24.31 %) and LDL-C (29.7-30.2 %). Moreover, the HDL-C levels were higher in the group administered with WSF-FM-572 in comparison with HCD group ($p < 0.05$). Likewise, triglycerides and NEFA levels were lower with the administration of WSF-572 ($p < 0.05$). Consequently, the cardiovascular risk indexes (atherogenic index of plasma and non-HDL-C/HDL-C ratio) were significantly lower ($p < 0.05$) with the WSF-572 treated group. Fecal lipid analysis revealed that cholesterol and NEFA were significantly higher in the group treated with WSF-572. However, not changes were observed in fecal bile acids analysis ($p > 0.05$). Due to the excretion of cholesterol in feces, the inhibition of

micellar solubilization of cholesterol was evaluated in RP-HPLC peptide fractions from WSF-572. All fractions presented capacity for inhibiting cholesterol micellization. However, eight peptides were identified in the active fraction (F3) by LC-MS/MS. The characteristics of peptides, mainly hydrophobicity, may explain the inhibition of micellar solubilization of cholesterol and may be related to the increase of fecal excretion of cholesterol and exert therefore the hypocholesterolemic effect in rats. Henceforth, the results suggest that peptides may be associated to the hypocholesterolemic effect of fermented milks, specifically with *L. lactis* NRRL B-50572

INTRODUCTION

Hypercholesterolemia is a main modifiable risk factor related to the development of atherosclerotic cardiovascular diseases (CVD) (Saeed et al., 2015). The management of dysregulated cholesterol levels includes the pharmacological therapy and changes of the lifestyle according to diverse clinical guidelines. Functional foods and nutraceuticals are also recommended for the management of lipids since their hypolipidemic effect was also widely studied (Poli et al., 2018, Thongtan et al., 2022). There are several compounds that showed hypocholesterolemic effect (*v.g*. fiber, glucans, phytosterols, probiotics) (Chen et al., 2008). Fermented milks are widely reported with multiple health effects, including the hypocholesterolemic effect. Most evidence show that living bacteria (probiotics) may be responsible for the hypocholesterolemic effect *in situ*, which depends on the type of microorganism used for fermentation (Cho et al., 2015; Ziaei et al., 2021). Nevertheless, there is limited evidence that showed the hypocholesterolemic effect of fermented milks components.

A previous study demonstrated that the low-molecular weight fraction (< 10 kDa) from milk fermented with *Streptococcus thermophilus* exert hypocholesterolemic effect compared with complete fermented milk, whey, and casein fractions (Kawase et al., 2001). The results indicate differences in the hypocholesterolemic effect in the different fractions, and the low-molecular weight fraction (<10 kDa) exerted better hypocholesterolemic effect than the high-molecular weight fraction (>10 kDa). Henceforth, it was suggested that metabolites derived from fermentation are capable to reduce cholesterol levels. However, the metabolites responsible for this effect is still unclear. There are reports that indicate that fermented milks are rich in bioactive compounds such as bioactive peptides. These reports indicate that peptides released by the proteolytic system of lactic acid bacteria exert a wide range of beneficial effects. The biological effects of bioactive peptides include the antihypertensive, antioxidative, immunomodulatory effects (Jakubczyk et al., 2020). The hypolipidemic effect of milk-derived peptides is also reported. However, the obtention of these hypolipidemic peptides was using specific enzymes (*v.g*. pepsin, neutrase, trypsin) (Jiang et al., 2020, Nagaoka et al., 2001).

Hypocholesterolemic peptides are capable to regulate cholesterol levels by different mechanistic pathways such as the inhibition of micellar solubility of cholesterol and bile acid binding-capacity.

In this sense, the inhibition of cholesterol micellization by peptides decreases cholesterol absorption in the intestine (Boachie et al., 2018). Also, peptides capable to bind to bile acids decreases enterohepatic circulation of bile acids that results in the decreasing of cholesterol levels in bloodstream. This resulting in a negative feedback regulation of the novo synthesis of bile acids by the utilization of cholesterol as precursor (Boachie et al., 2018, Ngoh et al., 2017). In this sense, water-soluble fractions (WSF) from milks fermented with *Lactococcus lactis* NRRL B-50572 and NRRL B-50600 demonstrated through *in vitro* studies inhibit the cholesterol micelles formation and bind to bile acids after simulated gastrointestinal digestion of these fermented milks. Nonetheless, the in vivo effect of these WSF in a hypercholesterolemia model is still unknown. The aim of this study was therefore to evaluate the hypocholesterolemic effect by the administration of WSF (< 10 kDa) in hypercholesterolemic Sprague-Dawley rats and identify the possible peptides involved with the effect.

MATERIALS & METHODS

Preparation of Water-Soluble Fractions from Fermented Milks

Fermented milks with *L. lactis* NRRL B-50572 (572) and NRRL B-50600 (600) were prepared according to previously reported protocol (Rendon-Rosales et al., 2019). In summary, the *L. lactis* strains were cultured (1 % v/v) in M17 broth supplemented with lactose or dextrose. Fresh cultures were then inoculated (3 %v/v) in reconstituted skim milk (10 %w/v) previously heat-treated (85 °C / 30 min) and incubated during 12 h for *L. lactis* NRRL B-50572 and 24 h for *L. lactis* for NRRL B-50600. Next, these cultures were inoculated (3 % v/v) in milk prepared as previously described and the milks were incubated for 48 h. All incubations were carried out at 30 °C. After 48 h of incubation, the fermented milks were heat treated (75°C/15 min) and immediately cooled until achieve 4 °C. For obtain water-soluble fractions (WSF). Fermented milks were centrifuged (3600 xg, 40 min, 4°C) and the supernatant (crude extract) were recovered for further fractionation. Crude extract was transferer to a stirred ultrafiltration cell fitted with cut-off size membrane with molecular weight of 10 kDa. The permeate (WSF < 10 kDa) were recovered in sterile tubes for further use in the animal study.

Animal Study Protocol

Animal study was conducted in accordance with the Guide for Care and Use of Laboratory Animals and approved by the Bioethics Committee of CIAD, A.C. (approval no. CE/017/2019). Sprague-Dawley rats (male, 4 weeks age, body weight = 190.4 ± 7.3 g, n= 28) were purchased from Bioinvert (Mexico, City, Mexico). For three weeks before being used for experiments, rats were maintained under standard diet (Abene 7100 maintenance, Bioinvert, Mexico City, Mexico) and *ad libitum* purified water in specific conditions that consisted in 12 h light/dark schedule in a humidity (40-55 %) and temperature controlled (22-24 °C). After the acclimatation, samples of blood were collected from tail and further determination of serum total cholesterol. The cholesterol

levels and body weigh were used as variable for randomization using a simple randomization procedure (computerized random numbers).

Four experimental groups were formed (n = 7). Next, the treatments were assigned for each group (**Figure 1)** that consisted in 1) control group: standard diet + purified water, 2) Hypercholesterolemic control (HC): High-cholesterol diet (HCD) + purified water, 3) HCD + WSF-572 and, 4) HCD + WSF-600. The HCD diet was formulated with the following components: standard diet (70%), lard (20%), egg yolk (8%), cholic acid (0.5%) and cholesterol (1.5%). During five weeks rats daily received the treatments via oral gavage (10 mL of water or WSF/kg body weight). The body weight, food and water consumption were recorded in all experimental period. At the end of experimental period, rats were fasting and euthanized by anesthesia administration (pentobarbital) via intraperitoneal injection (75 mg/kg of body weight). Blood samples were obtained by intracardiac puncture and collected in heparinized tubes. For plasma obtention, blood was centrifuged (2500 x g, 15 min, 4 °C). Fresh feces were collected during the last three days of the euthanasia of animals was carried out.

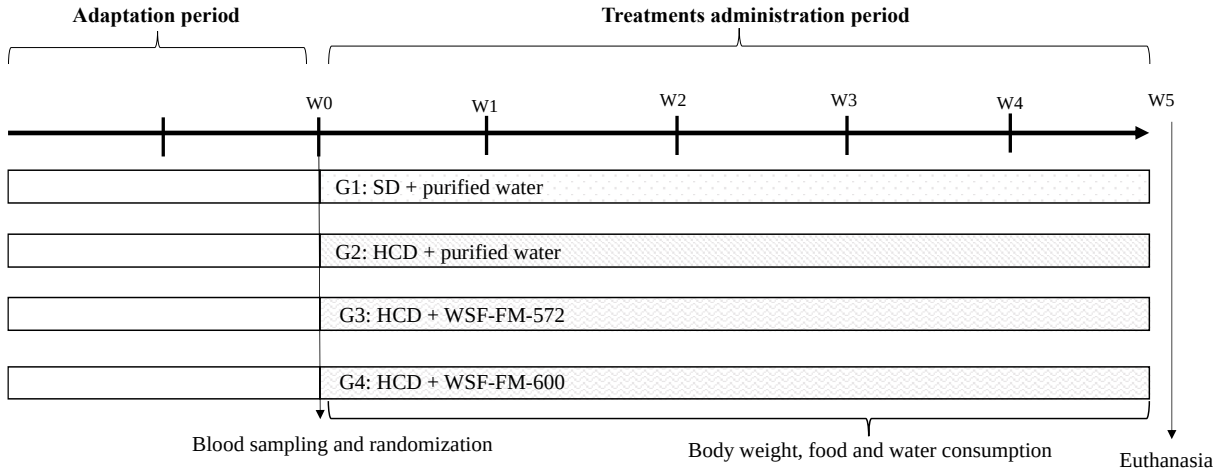

Figure 1. Experimental design of the study of the hypocholesterolemic effect of water-soluble fractions (WSF < 10 kDa) from milks fermented with *L. lactis* NRRL B-50572 (WSF-572) and NRRL B-50600 (WSF-572). SD: Standard diet; HCD: High-cholesterol diet.

Analysis of Lipid Levels in Plasma

Total cholesterol, LDL-C/VLDL-C, HDL-C and triglycerides levels were determinate in plasma using colorimetric or fluorometric enzymatic kit assay following the manufacturer's protocol (Cell Biolabs). For NEFA determination, lipids were extracted from plasma in according with previously report (Matyash et al., 2008). Briefly, plasma was mixed with methanol (1:3), next, methyl-tert-butyl ether (MTBE) was added, and the phase separation was induced by adding ultrapure grade

water. Upon 10 min of incubation a room temperature, the sample was centrifuged (1000 x g, 10 min), and then the upper phase was collected and dried by stream of nitrogen. Finally, the extracted dried lipids were dissolved in 1 % triton X-100 in the same initial volume of plasma for NEFA determination. NEFA was determinate using enzymatic colorimetric kit (Randox Laboratories, United Kingdom) according to the protocol of commercial kit.

Analysis of Lipids in Feces

NEFA and cholesterol were determinate in lipid extract from freeze dried feces. Briefly, lipid was extracted from feces according to protocol described by Mopuri et al., 2021 that consisted in the improved Folch method for tissue lipid quantification. Briefly, 100 mg of powder feces were homogenized with 12.5 ml of chroloroform:methanol (2:1) during two minutes. Homogenized samples were sonicated during five minutes and were incubated overnight in an orbital water bath shaker (450 rpm, 25 °C). Samples were centrifuged (3000 xg, 10 min) and the supernatant was collected and washed with NaCl solution (0.9 %) by centrifugation (2500 xg, 10 min). The supernatant was collected and washed in two successive steps of methanol (50 %) by centrifugation (2500 xg, 10 min). Then, the upper phase was discarded, and the lower phase was recovered and dried under a stream of nitrogen in a water bath (45 °C). Dried extracted lipids were suspended in 50 % ethanol with 1% w/v of triton X-100. Cholesterol and NEFA levels were determinate using enzymatic colorimetric kits (Cell Biolabs and Randox Laboratories, respectively) using the manufacturer´s protocol.

Determination of Bile Acids in Feces

Bile acids were extracted from freeze dried feces. 50 mg of powder feces were homogenized with ultrapure grade water for two minutes. Subsequently pure isopropanol was added to the mix for obtain final relation of 8:2 isopropanol:water. Next, then samples were sonicated during 30 min and further incubation at room temperature for 20 min. The samples were next centrifugate to 20000 xg for 20 min at 4 °C (Humbert et al., 2012). The supernatant was recovered and diluted in water for bile acids quantification using enzymatic colorimetric assay kit (Cell Biolabs) following the manufacturer's protocol.

Identification of Potentially Hypocholesterolemic Peptides

Isolation of Peptides Fractions by RP-HPLC

The WSF with the better hypocholesterolemic effect was selected for further identification of peptides by mass spectrometry. Peptide fractions were isolated by RP-HPLC (series 1200 Agilent technologies) equipped with an analytical scale-fraction collector. Peptide separation was conducted using C18 column (Agilent Zorbax Eclipse AAA, 4.6 x 150 mm, 3.5 µm particle size) using gradient elution. The phase mobile phase A consisted of 0.04 % v/v trifluoroacetic acid

(TFA) in water and mobile phase B consisted of 0.03% (v/v) TFA in acetonitrile. The following gradient program was carried out: 0-30 min, 0-60 % B; 30-35 min, 60-95 % B; 35-39 min, 95-100 % B; 39-40 min, 100-0 % B, and maintained for 8 min for baseline equilibration between injections. The flow rate was 0.250 mL/min and peptides were detected at 214 nm and the injection volume was set to 20 µL. Fifteen HPLC fractions were collected, freeze-dried, and finally resuspended in water for hypocholesterolemic evaluation.

Evaluation of Hypocholesterolemic Activity of HPLC Fractions

The inhibition of micellar solubility of cholesterol assay was used for identification of potential hypocholesterolemic activity of peptide fractions according to previously protocol (Kirana et al., 2005). Lipids with final concentrations of 0.5 mM cholesterol, 2.4 mM phosphatidylcholine and 1 mM linoleic acid were solubilized and mixed in methanol. The mixture was dried under nitrogen stream and the lipids were suspended in phosphate-buffered saline (15 mM, pH 7.4, 132 mM of NaCl) with 6.6. mM of sodium taurocholate. Next, the solution was sonicated for 20 min and incubated for 2 h at 37 °C. Thereafter, 100 uL of HPLC-fraction or 25 mg of cholestyramine as positive control was mixed with 0.5 mL of micellar solution. The mixture was sonicated once (2 min) and incubated for 2 h at 37 °C. The mixture was centrifuged at 10 000 xg during 10 min and the supernatant was recovered and filtrated through 0.22 µm syringe filters. The cholesterol in the filtrates was determinate using an enzymatic assay kit (Randox Laboratories). The percentage of inhibition was calculated according to the below equation. Additionality results also were expressed as efficiency of inhibition (IER), for this, protein content was determinate using DC Protein assay based in Lowry method (Biorad).

$$\textit{Inhibition of MSC (\%)} = [C_0 - C_S/C_0)] \times 100$$

$$\textit{IER} = \textit{inhibition of MSC (\%) / protein content (mg/mL)}$$

Where C_0 represents the cholesterol content in supernatant without sample; C_s is the cholesterol content in the supernatant of micelles treated with sample.

Analysis of Peptides by Mass Spectrometry (LC-ESI-MS/MS)

HPLC-fractions were lyophilized and suspended in MS grade water for MS/MS analysis. For peptide analysis, HPLC-fractions were injected in a nano-liquid chromatography couplet to mass spectrometry (nano-LC-MS/MS) system (1100 series LC/MSD trap, Agilent technologies, Waldbronn, Germany). Peptides (2 µL of injection) were separated in a nanocolumn (150 mm × 0.75 µm, 3.5 µm; Agilent Technologies) in a gradient elution The gradient was based on the increment of solvent B (water-acetonitrile-formic acid, 10:90:01) in solvent A (water-acetonitrile-formic acid, 90:10:01) as follows: 0-10 min, isocratic (3%B); 10-20 min, 3-45%B; 30-33 min, 45-65%B; 33-35 min, 65-97% and 35-37 min, 97-3% B with a flow rate of 0.7 µL/min. Peptides were ionized using electrospray interface and nitrogen was used as nebulizing and drying gas at a flow

rate at 6.0 L/min at 100 °C, and the needle voltage was set at 4kV. Mass spectrometer was operated in data-dependent analysis (DDA) in a positive ion mode with acquisition time of 150 ms. The isolation of ions was for MS1 (100-2200 m/z) and MS2 (100-1200 m/z). The energy of collision used for fragmentation was 0.30 V. The signal threshold was 30,000 in auto MS analysis. Peptide identification was perform using the search engine Mascot server against SwissProt and UniProt, restricted to mammalian or bovine proteins. The search parameters were: semitrypsin as the cleavage enzyme; fixed modification, none; variable modification, none; precursor mass tolerance was set to 1.2 Da and fragment mass tolerance set to 0.5 Da. Peptide charge was set at +1, +2 and +3 in monoisotopic mass value.

Additional *in silico* analysis of peptide sequences such as *in silico* digestion was performer using Expasy-Peptide Cutter (www.expasy.org), Bioactivity score was carried with PeptideRanker (distilldeep.ucd.ie/PeptideRanker/), physicochemical properties of peptides were performed using PepDraw (www2.tulane.edu/~biochem/WW/PepDraw). Hydrophobicity composition was analyzed using (Peptide 2.0 (www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php), and finally BIOPEP database was used for search sequences with reported biological activities (Minkiewicz et al., 2019).

Statistical Analysis

All data are expressed as mean ± error standard (SEM). Normality test (Shapiro-Wilk test) was performed before the analysis of variance (ANOVA) and mean comparison was carried out using Fisher test for multiple comparison. The significance was defined when $p < 0.05$. All statistical analysis were performed using the GraphPad Prism software. Correlation analysis was carried out using corrplot running in R studio using Pearson coefficient.

RESULTS AND DISCUSSION

Baseline Clinical Characteristic of Rats

Baseline clinical characteristic of Sprague-Dawley rats are summarized in **Table 1**. Total cholesterol and body weight were used as variables for randomization. No significant differences were found in total cholesterol ($p= 0.957$) and body weight ($p=0.874$) among the experimental groups. Once the rats were randomly assigned to groups treatments were started. High-cholesterol, high-fat, high-cholate diet was used for inducing hypercholesterolemia in the murine model. It has been previously reported that a atherogenic diet that consisted mainly of cholesterol and cholate is effective in inducing hypercholesterolemia in rodents, in spite the fact that the rats are resistant to hyperlipidemia. In this study the diet was enriched with lard and egg yolk as a source of dietary cholesterol in order to induce hypercholesterolemia (Cunha et al., 2021). Fat content and cholesterol content (determined by gas chromatography) were 33.59 % and 3.17 % w/w, respectively. Previous studies indicate that cholesterol content above 1.5 % was considered as

hypercholesterolemia. However, studies of dietary models for hypercholesterolemia indicate that, hypercholesterolemia with high cardiovascular risk factor coupled with increase of serum LDL-C, also can be reflected in the reduction of HDL-C (Matos et al., 2005). Thus, based in previous studies, a high cholesterol diet with a high fat content is effective for inducing hypercholesterolemia in rats.

Table 2. Baseline clinical characteristics of Sprague-Dawley rats

	Control	**HC control**	**WSF-572**	**WSF-600**	***p value***
Total cholesterol	68.02 ± 10.28	65.47 ± 16.77	64.71 ± 14.01	63.59 ± 19.98	0.957
Body weight	351.2 ± 28.4	360.8 ± 32.6	356.7 ± 35.4	364.5 ± 35.46	0.874

Body Weight Gain and Food Intake

During five weeks of the experimental period the body weight gain of the rats was recorder (**Figure 2A**). All experimental groups increased body weight gain. Nevertheless, groups with HCD diet significantly increased body weight gain in all the experimental period compared with the control group (standard diet). In fact, this increase of body weight gain was observed since one week of consumption of HCD. At the end of the experimental period, body weight gain was 34.37 % more in the HC control compared to the standard diet control. Likewise, changes of body weight gain during all experimental weeks were determined an expressed as area under the curve (AUC). HCD remarkably increased body weight gain by above 2.5-fold compared with the control (**Figure 2B**). The increase of body weight gain was due to the excess of calories from dietary fat. Therefore, it was reflected in the increase of fat tissue deposit (Fidèle et al., 2017). Specifically, previous reports indicated that fat deposit was characterized by large adipocytes couplet to adipose inflammation leading to the development of obesity (Poret et al., 2018). It was also reported that cholesterol, induced the synthesis of specific hormones (*e.g.*, cortisol, testosterone, estrogens) which contributed to the increase of body weight (Fidèle et al., 2017). WSF from fermented milks showed effect in the reduction ($p < 0.05$) of body weight gain at week five by 36.5 % compared to the HC control. (**Figure 2A**). Furthermore, the AUC values showed that the administration of WSF-572 was most effective in regulating body weight gain during all the experimental period.

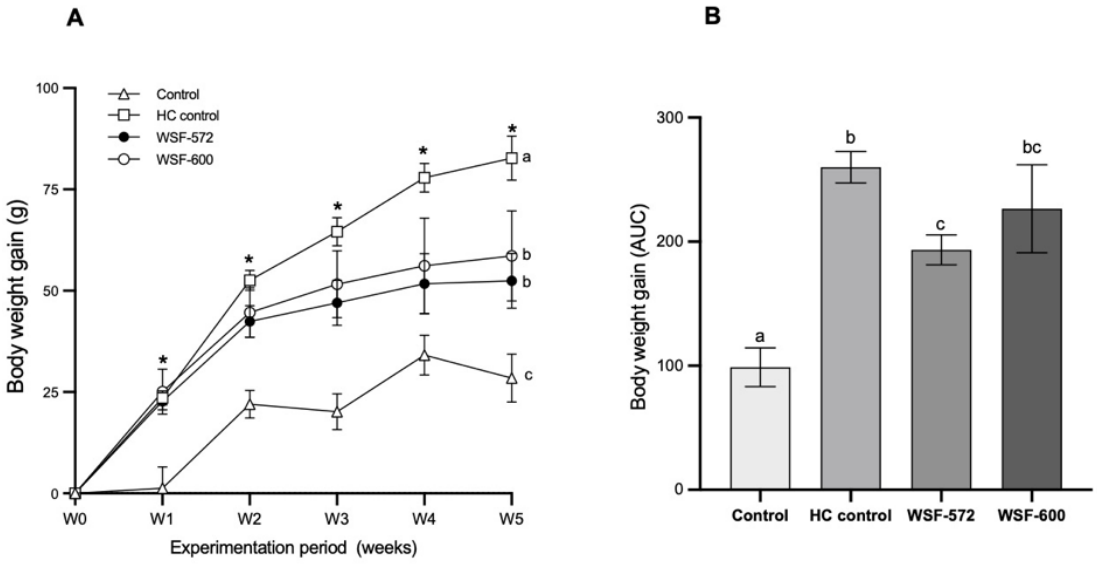

Figure 2. Body weight gain during the administration of water-soluble fractions (WSF) derived from milks fermented with *L. lactis* NRRL B-50572 (WSF-572) or NRRL B-50600 (WSF-600). Asterisks or literals indicate (*) significative differences ($p < 0.05$) among experimental groups. Data represent the mean (n=7) ± standard error (SEM).

To know the impact of HCD in body weight gain, the average food intake was determined. **Figure 3** depicts the behavior of food intake during all experimental periods. In all weeks, food intake in the groups with HCD was less compared with the standard diet group (control). Likewise, the administration of WSF did not exert changes in food intake and was equal to the HC group ($p > 0.05$). In this experiment, the results of food intake were contrary to the expectations. Several reports indicated that the increase of body weight is accomplished by an increase of appetite, due to the appetite-stimulating hormone reflecting therefore in an increase of food intake (Fidèle et al., 2017, Marques et al., 2015, Licholai et al., 2018). The decrease in food intake in the high-fat diet was also reported by Shinohata et al., 2022, however body weight gain was not observed in rats after a high-fat diet consumption. These results can be explained by different mechanisms, since it has been reported that the relationship of food intake and weigh gain depends on a homeostatic mechanism that regulates daily caloric intake, a hedonic factor (palatability) and cognitive factors (Pandit et al., 2012; Kenny, 2011; Licholai et al., 2018).

The regulation of body weight gain by the administration of WSF was therefore independent of the food ingested. Bioactive compounds with antiobesity effect exerted by multiple mechanisms that includes the balance energy intake, influence on lipid metabolism, effect on adipocytes and the modulation of gut microbiota were reported. Bioactive peptides are part of the major compounds released after the fermentative milk process. The inhibition of key enzymes on lipid metabolism and inhibition of adipose cell differentiation could explain less body weight gain by peptides presents in the WSF. Previously, peptides derived from fermented milk (*Limosilactobacillus*

fermentum) were effective in inhibiting pancreatic lipase (Manzanarez-Quin et al., 2023). The inhibition of this enzyme resulted in low fatty acids absorption, reducing therefore the energy intake and a less body weight gain. Also, peptides can modulate adipose tissue differentiation by decreasing the expression of mRNA of adipogenesis related factors such as peroxisome proliferator-activated receptor gamma (PPARγ) and sterol regulatory element binding protein 1c (SREP-1c) in 3T3-L3 cells (Singh et al., 2022, Manzanarez-Quin et al., 2023). Peptides from milk showed to suppress appetite and thus prevent calorie intake and weight gain (Park & Nam, 2015). Nevertheless, in this study, WSF did not exert an effect in food intake compared to the HC control group. Thus, these results evidenced that WSF exerted a positive effect in the management of body weight gain independently of the amount of food ingested.

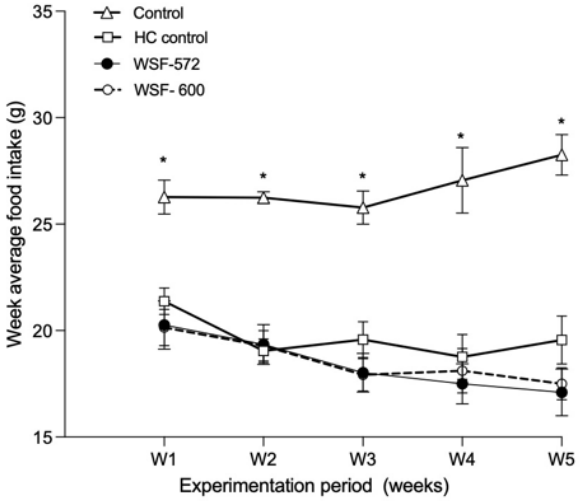

Figure 3. Food consumption (g) during the experimental period (five weeks) of hypercholesterolemic Sprague-Dawley rats treated with water-soluble extracts derived from milks fermented with *L. lactis* NRRL B-50572 (WSF-572) or NRRL B-50600 (WSF-600). Asterisks indicate (*) significative differences ($p < 0.05$) among experimental groups. Data represent the mean (n=7) ± standard error (SEM).

Effect of Administration of WSF Derived from Fermented Milks with *L. lactis* in Plasma Lipid Levels and Cardiovascular Risk Indexes

Previously studies demonstrated that milks fermented with specific lactic acid bacteria exerted a hypocholesterolemic effect. Particularly, milks fermented with *L. lactis* NRLL B-50572 and NRRL B-50600 showed hypolipidemic effect in Spontaneously hypertensive rats (SHR) or in hypercholesterolemic rats. However, it was unclear if the low-molecular fraction (< 10 kDa) of these fermented milks was capable of exerting a hypocholesterolemic effect *in vivo*. Therefore, based in a hypercholesterolemic rat model, the WSF (< 10 kDa) were evaluated. **Figure 3** illustrates the effect of the WSF on plasma lipid levels. As expect, total cholesterol as well as LDL-C/VLDL-C remarkably increased up to two-fold and four-fold, respectively, ($p < 0.05$) in the HC control

group compared to the control group after five week consumption of HCD. Conversely, HDL-C significantly decreased by 50 % ($p < 0.05$) in the HC control.

Rats are characterized by the high content of HDL-C and low levels of LDL-C, nonetheless, hypercholesterolemia in rats, elevated LDL-C is coupled with lower HDL-C (Wang et al., 2015; Lee et al., 2019). These cholesterol levels changes indicate alteration in cholesterol metabolism, which is associated to the expression of genes related to lipid metabolism, such as SREPB-2 and HMGCR (Wang et al., 2015). Also, the low levels of HDL-C are related to alterations of lecithin cholesterol acyltransferase (LCAT) activity, since this enzyme catalyzes free cholesterol esterification for the efflux of cholesterol by HDL particles, in this sense, LCAT deficiency results of low HDL-C levels (Rousset et al., 2011). Interestingly, triglycerides levels were unaffected after HCD consumption. Triglycerides levels in bloodstream depend on lipoprotein lipase (LPL) activity (Wung et al., 2006). LPL hydrolyzes triglycerides from chylomicrons and VLDL, however, this enzyme is increased in hyperlipidemia conditions and atherosclerotic lesions (Pentikäinen et al., 2022). Higher activity of LPL results in a high content of fatty acids (NEFA) (Tsutsumi et al., 2003). In this study, NEFA content was slightly increased in the HC control group with is consistent with previous reports (Zhang et al., 2015).

The administration of WSF derived from fermented milks exerted a positive effect in the regulation of plasma lipid levels in hypercholesterolemic rats (**Figure 4**), since both WSF were capable to reduce total cholesterol in a range from 21.7 to 24.8 % ($p < 0.05$). Likewise, LDL-C/VLDL-C levels were 29-7-30.2 % lower ($p <0.05$) in both WSF treatments with respect to the HC control ($p < 0.05$). Furthermore, the group treated with WSF-572 showed higher levels of HDL-C (not significant) in all groups with HCD-C. Moreover, only WSF-572 significantly reduced triglycerides levels ($p < 0.05$) and NEFA content in plasma ($p < 0.05$) by 35 % and 31.72%, respectively. The positive effect of the regulation of lipid levels by WSF resulted in the reduction of cardiovascular risk indexes. **Figure 5** depicts indexes determined as atherogenic index of plasma (AIP) and non-HDL-C/HDL-C ratio. The ratios of atherogenicity are positively correlated with the development of coronary heart disease (Niroumand et al., 2015). As expect, the HC group showed markedly elevated atherogenic indexes values ($p < 0.05$) increasing by eight and ten-fold for AIP and non-HDL-C/HDL-C ratio, respectively. The administration of WSFs and their hypolipidemic effect was evidently reflected in the decrease of atherogenicity indexes. Particularly, due the better effect of WSF-572 in the decreased levels of triglycerides and increased levels of HDL-C, this WSF significantly decreased ($p < 0.05$) the risk of the development of atherosclerosis and related diseases.

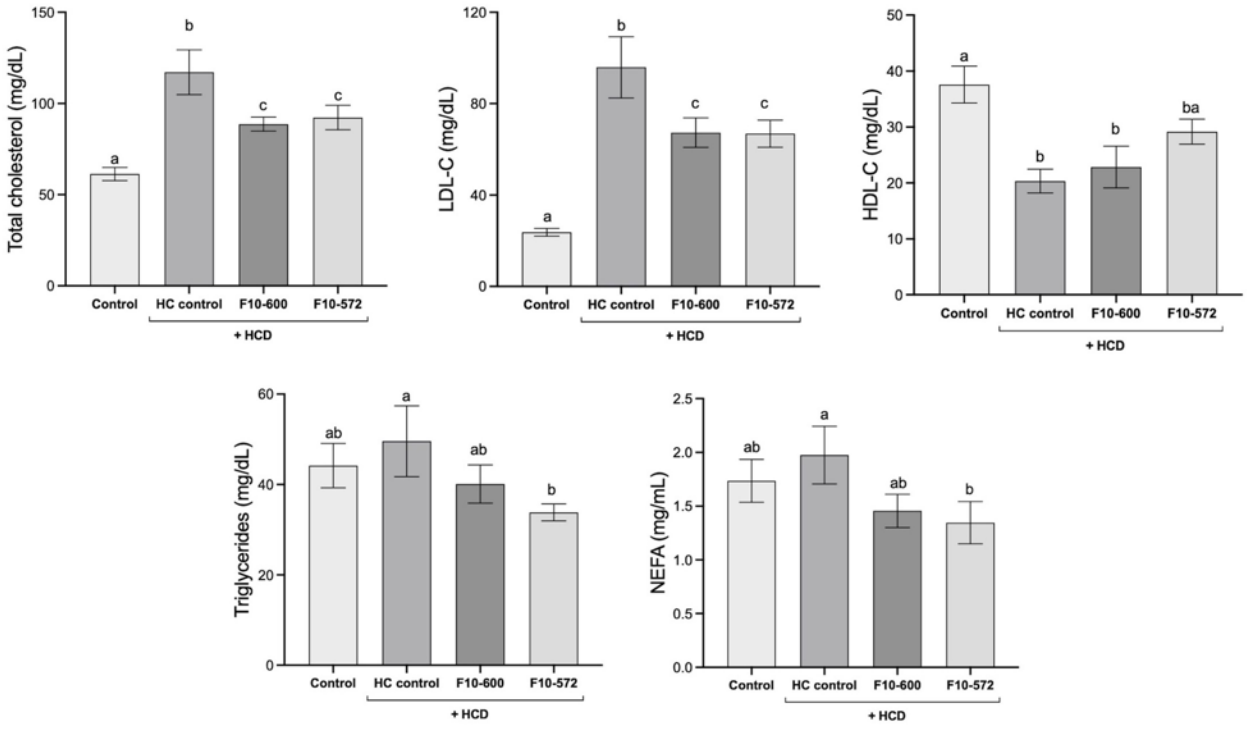

Figure 4. Effect of the administration of water-soluble fractions derived from fermented milks with *L. lactis* NRRL B-50572 (WSF-572) and NRRL B-50600 (WSF-600) in lipid profile in plasma (total cholesterol, LDL-C/VLDL-C, HDL-C, triglycerides and NEFA) of hypercholesterolemic Sprague-Dawley rats. Data are presented as mean (n=7) ± standard error (SEM). Literals indicate statistical differences between treatments.

The analysis of plasma lipids and atherogenic index reveal therefore that WSF exerted hypocholesterolemic effect and this effect may be related to the metabolome produced during the fermentation of milk with *L. lactis*. Previously fermented milks with L. *lactis* NRRL B-50572 or NRRL B-50600 showed hypocholesterolemic effect, unlike other studies, these bacteria were non-probiotic, and thus the effect may be associated to bioactive compounds such as peptides. Additionality, peptide fractions derived from these fermented milks showed to inhibit cholesterol mixed micelles and bind to bile acids in vitro (Rendon-Rosales et al., 2019). Hence, these activities may explain the hypocholesterolemic effect in the present study. The inhibition of the formation of cholesterol micelles in the intestinal lumen resulted in a low absorption of lipids (dietary cholesterol), and an increased excretion in feces. Moreover, peptides with the capacity to bind to bile acids produce alteration in the enterohepatic circulation of bile acids resulting in the loss of bile acids (fecal excretion) and *de novo* synthesis of bile acids from cholesterol, decreasing therefore the cholesterol levels. (Boachie et al., 2018). However, peptides capable to cross the intestinal barrier can modulate cholesterol metabolism either in the enterocyte or in the hepatocyte. In these cells, peptides can decrease cholesterol biosynthesis thought the inhibition of the enzyme

hydroxymethylglutaryl-Coenzyme A (HMG-CoA) reductase. Inhibition of HMG-CoA reductase causes intracellular depletion of cholesterol, increasing the cell uptake of LDL-C (Silva et al., 2021). The enzymatic activity of HMG-CoA reductase also can be modulated by the regulation of transcriptions factors. Evidence suggests that specific hypocholesterolemic peptides regulate this activity by the sterol regulatory element binding protein 2 (SREBP2) in HepG2 cells (Kumar et al., 2019).

Furthermore, peptides are capable to increase the catabolism of LDL-C by the induction of the expression of LDL-receptors (LDLR) (Boachie et al., 2018). In this study, HDL-C levels were slightly enhanced by the administration of WSF-572. Therefore, this observation suggested that compounds in the WSF-572 could modulate the reverse cholesterol transport (RCT) which is mediated by HDL. The HDL-C levels can be explained mainly by the activity of proteins LCAT, acyl-coenzyme A: cholesterol acyltransferase (ACAT) and ATP-binding cassette, sub-family G, member 1 (ABCG1) (Marques et al., 2018). In fact, ABCG1 may explain the 30% of HDL-C levels. Recent studies evidenced that the dipeptide FP tended to increase hepatic ABCG1 in rats coupled to an increase of HDL-C levels (Banno et al., 2019). Conversely, the modulation activity of ACAT or LCAT through bioactive peptides is still limited. Therefore, the slight increase of HDL-C levels may be explained by the enhanced efflux of cholesterol by the RTC process, decreasing the cholesterol accumulation in tissues. Nevertheless, further molecular studies are need in order to elucidate the underlaying mechanisms.

The triglycerides-lowering effect of bioactive peptides by multiple mechanism was reported (Nagaoka, 2019). The inhibition of pancreatic lipase leads to lower the absorption of monoglycerides and fatty acids, resulting in a low concentration of triglycerides. Also, it is possible that the activity of LPL and hepatic lipase may also be enhanced by WSF-572. In fact, specific peptides increased hepatic lipase (e.g., VVVP) resulting in low levels of triglycerides (Kagawa, et al., 1996). Notwithstanding, that when lipase activity increased, an increase of NEFA was also observed. In this study, NEFA levels were evidently decreased when the WSF-572 was administered. Hence, it possible that other mechanisms were involved. Peptides have been showed to inhibit fatty acid synthase (FAS), decreasing the biosynthesis of long-chain fatty acids. For example, KNPQLR, EITPEKNPQLR, and RKQEEDEDEEQQRE derived from β-conglycinin inhibit FAS and decreased lipid accumulation in 3T3-L1 adipocytes. (Martinez-Villaluenga et al., 2010). In addition, water-soluble extracts (< 3 kDa) from fermented milk (*Limosilactobacillus fermentum*) decreased lipid accumulation in the 3T3-L1 adipocytes (Manzanarez-Quin et al., 2023). It has been reported that the inhibition of FAS in hypothalamus and pancreatic beta cell exerts protective effect against high-fat diets and metabolic syndrome (Chakravarthy et al., 2009). It is noteworthy to note that the reduction of body weight coincides with reduction of body weight after WSF-572 administration. This finding could be associated to the increase of energy expenditure and β-oxidation by the activation of peroxisome proliferator activated receptor alpha PPARα). Specific peptides (e.g., YPFVV) showed reduce triglycerides by this mechanism (Yamada et al., 2012). More studies are needed for elucidate the mechanism involved in the

hypolipidemic effect of WSFs.

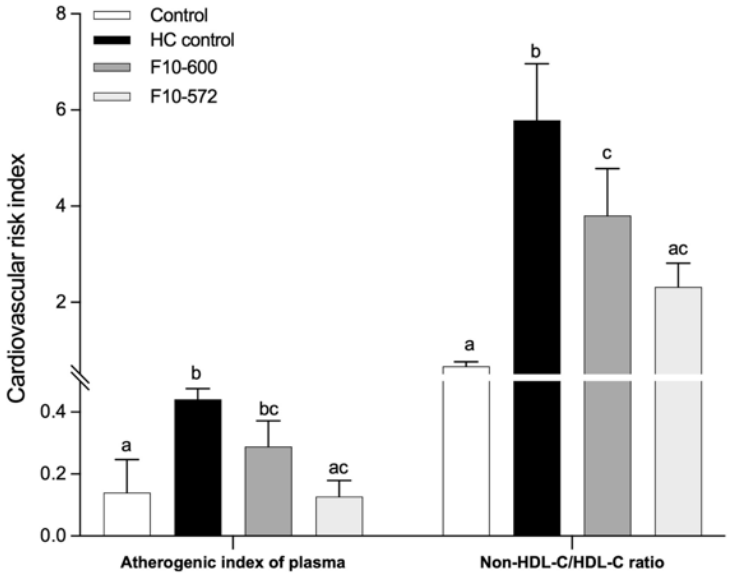

Figure 5. Atherogenic risk indexes expressed as atherogenic index of plasma (AIP) and LDL-C+VLDL-C/HDL-C ratio. Data are presented as mean (n=7) ± standard error (SEM). Literals indicate statistical differences between treatments for the same type of index.

Effect of the Administration of WSF Derived from Fermented Milks on Lipid and Bile Acids Excretion

The excretion via fecal lipids is a consequence of diverse mechanisms involved in the hypocholesterolemic effect of bioactive peptides. In this study, the lipids (cholesterol, NEFA) and bile acids excretion were evaluated. **Figure 6** depicts the lipids excretion by the effect of administration of WSF obtained from fermented milks. The excretion of lipids was markedly higher in all groups with intake of HCD. However, cholesterol was significantly ($p < 0.05$) more excreted in the group administered with WSF-572. This result coincides with previous reports that indicated that the hypocholesterolemic effect was associated to cholesterol excretion by the intestinal regulation of cholesterol absorption. (Lee & Youn, 2020). It this respect, peptides can inhibit cholesterol absorption through different mechanisms. The inhibition of cholesterol micelles is highly associated with a higher cholesterol absorption. In fact, peptide fractions derived from milks fermented with *L. lactis* NRRL B-5052 and NRRL B-50600 showed to inhibit cholesterol micelles (Rendon-Rosales, et al., 2019). The regulation of cholesterol absorption is also mediated by Nieman -Pick C1 like 1 (NPC1L1) protein transport (Duan et al., 2022). In this sense, specific peptides can bind to NPC1L1 and inhibit cholesterol absorption (Real & Gonzalez de Mejia, 2017), and therefore the non-absorbed cholesterol is excreted in feces. Moreover, the ATP-binding cassette subfamily G member 5 and 8 (ABCG5/8) sited in the enterocytes (brush border membrane)

promotes efflux of cholesterol from the enterocyte back into intestinal lumen for fecal excretion (Caponio et al., 2020). Casein-derived peptides demonstrated to reduce cholesterol levels in mice through the induction of trans-intestinal cholesterol excretion by the expression of ABCG5/8 (Lee & Youn, 2020).

In this study, coupled to cholesterol excretion, NEFA was also slightly excreted by the administration of WSF-572 in rats ($p < 0.05$) compared to the HC control. This result may reinforce the fact that peptide fractions decrease the micellization of lipids in the intestinal lumen, decreasing their absorption, which may be reflected in lower levels of plasma NEFA in our findings. In the other hand, bile acids were slightly more excreted with the WSF-600 treatment (not significant). A higher excretion of bile acids is associated to peptides capable of binding to bile acids (bile acid sequestrants). The hypocholesterolemic action occurs when peptides reduce the absorption of bile acids in the terminal ileum. As a consequence of this, de novo bile acids are then synthetized from cholesterol as precursors by the induction of activity of cholesterol 7-alpha-hydroxylase (CYP7A1), and thereby decreasing the cholesterol levels in the bloodstream (Boachie et al., 2018). In fact, a previous study (Rendon-Rosales, et al., 2019) peptide fractions derived from fermented milk with *L. lactis* NRRL B-50600 exerted capacity to bind to bile acids (cholic and taurocholate). Although the excretion of bile acids was small, peptide fractions with bile acid binding capacity could be partially responsible for the cholesterol-lowering effect of WSF-600. Collectively, the results obtained in this study suggested that WSF can regulate the intestinal absorption of lipids and thus regulate cholesterol levels in plasma.

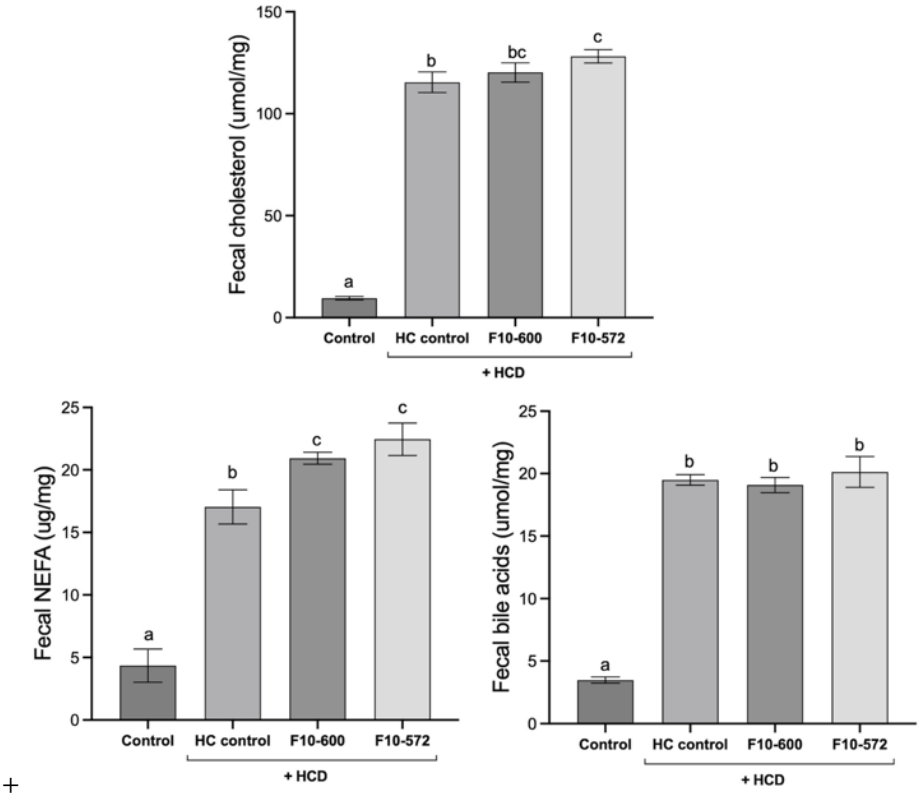

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Figure 6. Effect of water-soluble fractions derived from fermented milks with L. *lactis* NRRL B-50572 (WSF-572) or *L. lactis* NRRL B-50600 (WSF-600) on cholesterol, non-esterified fatty acids (NEFA) and bile acids excretion in hypercholesterolemic Sprague-Dawley rats. Data are presented as mean (n=7) ± standard error (SEM). Literals indicate statistical differences between treatments.

Evaluation of Potential Hypocholesterolemic Peptides

In order to know if peptide fractions were possibly involved in the hypocholesterolemic effect, the WSF was further fractionated in order to obtain peptide fractions by RP-HPLC. Based in the results in this study, WSF-572 was the fraction with the highest hypolipidemic activity. Also, cholesterol was higher excreted in feces. Thus, the inhibition of micellar solubilization of cholesterol was the mechanism possibly involved in the hypocholesterolemic activity. In this regard, WSF-572 was selected for further peptide fractionation for the evaluation of inhibition of micellar solubilization of cholesterol and finally to identify the peptides involved in the activity. Fifteen HPLC fractions were collected (**Supplemental Figure 1)** and each one of them was evaluated for its capacity to inhibit the micellar solubility of cholesterol. **Figure 7 A** depicts the hypocholesterolemic activity of the HPLC fractions from WSF-572. The positive control (cholestyramine), as expect, inhibited 77% of the micellar solubility of cholesterol. Conversely, all HPLC fractions inhibited cholesterol micelles in a range of 4.65 to 43.5%. Two fractions (F3 and F4) were the most active, showing the higher percentages of inhibition (above of 40 %, $p < 0.05$).

Based in protein concentration, the inhibitory efficiency was determined (**Figure 7B**). Results indicated that fraction 3 showed the highest efficiency ($p < 0.05$), followed by fraction 4, 7 and 6. Henceforth, peptides were identified in these HPLC fractions by MS-MS. In general, most studies have reported the hypocholesterolemic activity of hydrolysates derived from plant and animal proteins, and specifically from soy proteins (Caponio et al., 2020). In dairy products, the hypocholesterolemic peptides have been obtained by using specific proteases such trypsin, pepsin and neutrase (Nagaoka et al., 2001, Jiang et al., 2020). However, to the best of knowledge, there are limited studies that have evaluated the hypocholesterolemic activity of peptide fractions derived from fermented milks. In this regard, Kawase et al., 2000 showed that the molecular weight fraction (<10 kDa) derived from *Streptococcus thermophilus* TMC1543 exerted hypocholesterolemic activity, nonetheless the compounds involved in the effect were no identified. In this study, peptides could be related to the hypocholesterolemic effect, and its structures are discusses ahead.

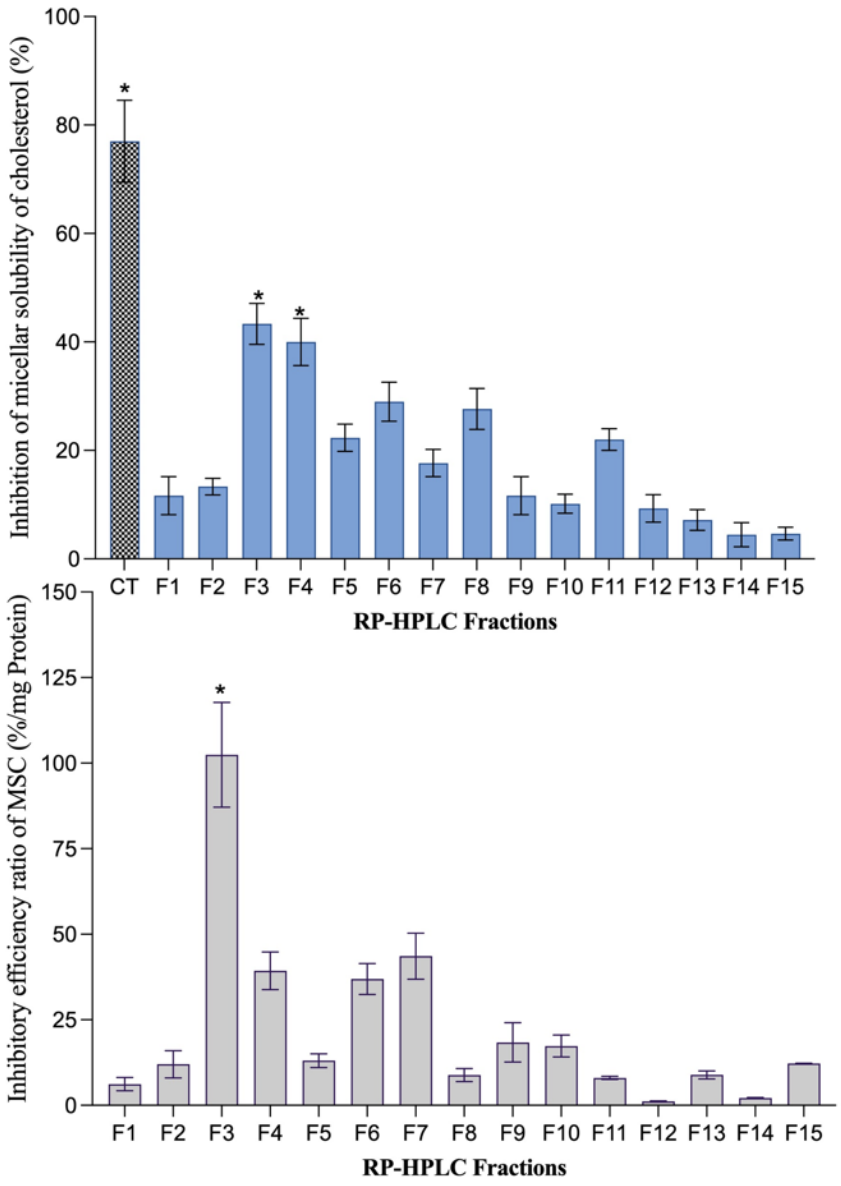

Figure 7. Inhibition of micellar solubility of cholesterol by collected RP-HPLC fractions (1-15) derived from WSF-572. Inhibitory activity is expressed as percentage of inhibition (**A**) and inhibition efficiency (B). Cholestyramine (CT), positive control. Asterisks (*) indicate statistical differences ($p < 005$) among fractions. Data represent the mean (n=3) with standard deviation.

Identification of Peptides with Potential Hypocholesterolemic Effect

A total of twenty-seven peptides were identified in WSF-572 (**Supplemental Table 1).** Identified peptides in WSF-572 showed molecular weights ranging from 477.76 to 1826.72 Da, and peptide lengths from 4 to 16 amino acids. Moreover, peptides present in the active fraction (F3) were shown

in **Table 2**. Overall, eight peptides were identified in the active fraction (F3) and most of them presented molecular weight ranging from 710 to 933.02 Da. The physicochemical properties involved in the hypocholesterolemic effect of these peptides were also investigated. Hydrophobicity is an important property associated to peptides capable to inhibit the micellar solubilization of cholesterol, since these peptides, compete with cholesterol to form micelles. Also, these peptides can interact with cholesterol or bile acids in the non-polar region. (Kimikazu et al., 1986, Chen et al., 2021).

Table 1. Identified peptides in the active HPLC-fraction (F3) from the water-soluble fraction derived from milk fermented with *L. lactis* NRRL B-50572 (WSF-572)

Exp. Mass	Ion m/z	Protein fragment	Sequence	pl	Hydrophobicity[a]/(% hydrophobic AA)	In silico digestion
1716.1055	859.06 (+2)	β-CN (f194-209)	QEPVLGPVRGPFPIIV	6.84	10.39 (68.7)	QEPVL,GPVR,GPFPIIV
933.0382	312.0 (+3)	β-CN (f184-191)	DMPIQAFL	2.95	8.2 (75)	DMPIQA,F,L
727.5382	243.5 (+3)	β-CN (f199-205)	GPVRGPF	10.9	10.12 (57.1)	GPVR,GPF
930.3927	311.1 (+3)	Lactrotransferrin (f386-394)	KGEADALNL	4	18.47 (44.4)	K, GEADA, L,N,L
677.9327	678.94(+1)	Lactrotransferrin (f203-209)	ADGVAFV	3.13	11.06 (71.4)	ADGVA,F,V
764.6782	255.90 (+3)	Serotransferrin (f599-605)	DKATCVE	4	18.24 (28.4)	DK, ATCVE
726.1695	243.06 (3+)	Seroalbúmin (f420-426)	VPQVSTP	5.69	8.74 (57.4)	VPQVSTP
710.7582	356.39 (2+)	Seroalbúmin (f438-444)	VGTRCCT	8.21	10.86 (14.2)	VGTR, CCT

Overall, peptides present in the active fraction showed a medium-high degree of hydrophobicity (**Table 2**), since their composition in hydrophobic amino acids residues were above 50%, specifically, sequences QEPVLGPVRGPFPIIV, DMPIQAFL and ADGVAFV presented more than 60% of hydrophobic amino acids. Additionality, Imai et al., 2021 found that peptides with at least two aromatic and two acidic amino acids showed relatively high micelle disruption activity. Nevertheless, in this study only one peptide (NICNISCDKFLDDDLT) showed this property. Thus, the inhibition of solubility of cholesterol may be explained by the presence of hydrophobic amino acids. In addition, as mentioned previously, peptides with the capacity to bind to bile acids in the intestinal lumen can decrease serum cholesterol levels by increasing the activity of CYP7A1 as well as disrupt cholesterol micelle formation. In this regard, Lapphanichayakool et al., (2017) indicated that the isoelectric point (pI) of peptides is another property of hypocholesterolemic peptides. Specifically, peptides with bile acid-binding capacity have pl in a range from 4 to 12.

Since pH affects net charge of side-chain amino acids of peptides in a specific pH, it also can affect the hydrophobicity or solubility of peptides. In this study, twenty peptides presented pI ranging from 4 to 11.56. Moreover, it has been described that Trp, Tyr, Phe, Leu and Val are present in bile acid binding peptides. In our results, seven peptides presented at least three of these amino acid residues that may also inhibit the solubilization of cholesterol micelles.

Furthermore, hypocholesterolemic peptides have been involved in inhibiting the biosynthesis of cholesterol either in the enterocytes or in the hepatocytes. In this regard, these peptides need to pass to the gastrointestinal tract and be absorbed in the mucosa and enterocytes. Thus, these barriers include mainly proteases and the intestinal epithelium (Renukuntla et al., 2013). In this sense, an *in silico* analysis was performed in order to predict gastrointestinal hydrolysis. The peptides released after in silico hydrolysis is presented in **Table 2** and **Supplemental table 1**. All peptides were partially hydrolyzed except for TCVADES and AESISSSE. Previous reports indicate that the inhibition of HMG-CoA reductase decreases cholesterol levels, furthermore, molecular docking studies indicated that the presence of Phe at the C-terminus is important for the inhibitory activity of peptides. In this study, four peptides (PEWVCTTF, GPVRGPF, SSAYSRGVF and RGVF) were identified with this property.

Nonetheless, after *in silico* hydrolysis, forty-two peptides were generated of which three peptides (GPF, GVF, ENF) presented Phe in the C-terminus. In fact, GPF and GVF exhibited high probability to be bioactive according to the Peptide Ranker tool (Score > 0.8). It is important to mention that these tripeptides have a high probability to be absorbed by PepT1 transporter (Renukuntla et al., 2013). Moreover, the presence of branched-chain amino acids (Val, Ile, Leu), mainly in the N-terminus also contributed to the hypocholesterolemic effect of peptides (Wang et al., 2023) by increasing the expression of LDLR in liver cells. In our results, seven peptides presented either Val or Ile in the peptides after in silico hydrolysis and only three in intact peptides. Wherefore, the presence of specific amino acids (hydrophobic, aromatics and branched amino acids) may explain the hypocholesterolemic effect of peptides present in WSF-572. Furthermore, some peptides released after *in silico* digestion were reported to have biological activities (**Supplemental table 1**), such as DPPV-IV inhibitors (GPF, PM, PK, NR), ACE inhibitors (GPFPIIV, PYP, IR) and antioxidative capacity (IR).

CONCLUSION

It was concluded that the low-molecular weight fractions (< 10 kDa) derived from milk fermented with *Lactococcus lactis* NRRL B-50572 or NRRL B-50600 decreased total plasma cholesterol and non-HDL cholesterol (LDLV+VLDL). Specifically, the fraction from milk fermented with NRRL B-50572 reduces triglycerides and free fatty acids (NEFA). Altogether, this hypolipidemic effect decreased the atherogenic coefficient (non-HDL-C/HDL-C ratio) and the atherogenic index of plasma. The hypolipidemic effect of WSF-572 coincided with a higher excretion of cholesterol and free fatty acids (NEFA). Moreover, isolated peptide fractions from WSF-572 showed capacity to

inhibit the micellar solubility of cholesterol and eight peptides with high hydrophobicity were identified in the active fraction. Therefore, several of the identified peptides may be responsible for the hypocholesterolemic effect. Nevertheless, additional studies are need to elucidate the underlaying mechanism involved in the hypocholesterolemic effect of peptides.

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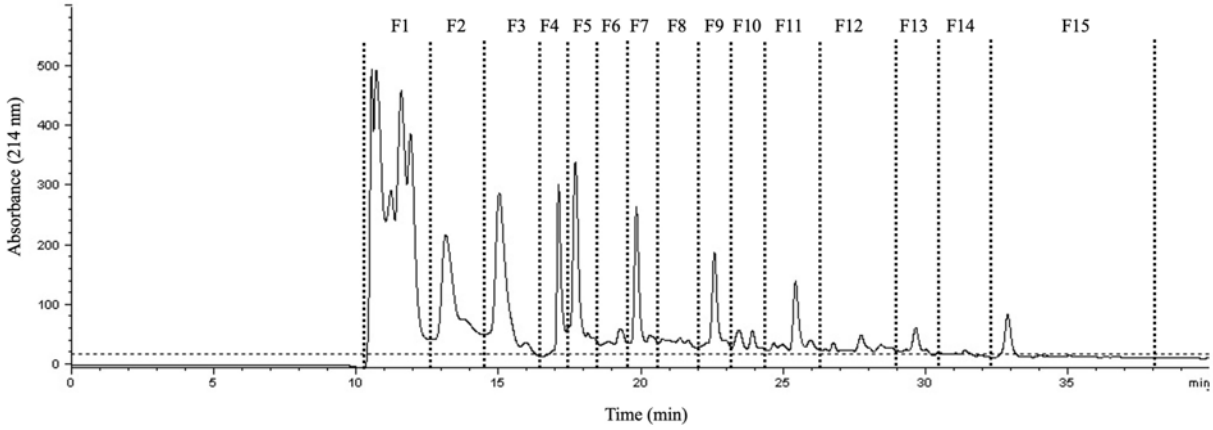
Supplementary Figure 1 (SF1)



SF1. RP-HPLC profile of peptides derived from water-soluble fraction from derived from milk fermented with *Lactococcus lactis* NRRL B-50572 (WSF-572). Chromatogram indicates the fractionation (F 1-15) for the assessment of the hypocholesterolemic activity based in the inhibition of micellar solubility of cholesterol.

Supplementary Table 1. Identification of peptides in the water-soluble extracts derived from

Lactococcus lactis NRRL B-50572 (WSF-2).

Experimental mass	Ion M/Z	Sequence	Protein fragment	Isoelectric point	Hidrophobicity (Kcal * mol -1)	Hydrophobic AA (%)	In silico hydrolysis
808.1437	405.1 (+2)	AESISSSE	αS1-CN (f62-69	2.92	16.38	25	AESISSSE
1755.6521	586.2 (+3)	EYGLFQINNKIWCK	α-La (f49-62)	8.78	12.73	35.71	E,Y,G,L,F, QINNK,I,W,CK
1069.7745	357.6 (+3)	TPTLNREQL	αS2-CN (f120-128)	6.58	13.1	33.33	TPTL,EQL, NR
981.2954	328.1 (+3)	PEWVCTTF	α-La (f25-31)	3.14	7.89	50	PEW, VCTT, F
723.6095	362.81 (+2)	TCVADES	Seroalbúmina (f58-64)	2.92	15.9	28.57	TCVADES
700.5682	234.53 (+3)	GACLLPK	Seroalbúmina (f180-186)	9	9.97	57.14	GAC, L, L, PK
862.3599	288.46 (+3)	LLKHKPK	Seroalbúmina (f537-543)	10.89	16.27	42.86	L, L, K, H, KPK
933.0382	312.0 (+3)	DMPIQAFL	β-CN (f184-191)	3.12	8.2	75	DMPIQA,F,L
727.5382	243.5 (+3)	GPVRGPF	β-CN (f199-205)	11.13	10.12	57.14	GPVR,GPF
930.3927	311.1 (+3)	KGEADALNL	Lactrotransferrina (f386-394)	4.01	18.47	44.44	K, GEADA, L,N,L
726.1695	243.06 (3+)	VPQVSTP	Seroalbúmina (f420-426)	5.23	8.74	57.14	VPQVSTP
710.7582	356.39 (2+)	VGTRCCT	Seroalbúmina (f438-444)	8.28	10.86	14.29	VGTR, CCT
677.9327	678.94 (+1)	ADGVAFV	Lactotransferrina (f203-209)	3.15	11.06	71.43	ADGVA,F,V
1716.1055	859.06 (+2)	QEPVLGPVRGPFIIV	β-CN (f194-209)	6.57	18.24	66.67	QEPVL,GPVR,GPFPIIV
764.6782	255.90 (+3)	DKATCVE	Serotransferrina (f599-605	4	18.24	28.57	DK, ATCVE
1826.7269	914.37 (+2)	NICNISCDKFLDDDLT	α-La (f71-86)	3.45	21.18	31.25	NICNISCDK, F, L, DDD, L, T
1108.9582	370.66 (+3)	KENFEVLCK	Serotransferrina (f563-571)	6.3	18.17	33.33	K, ENF, EV, L, CK
1054.6255	528.32 (+2)	KNYELLCGD	Serotransferrina (f225-233)	4	16.74	22.2	K,N,Y,E,L,L, CGD
898.8112	899.82 (+1)	DDQNPHSS	α-La (f63-70)	3.91	20.19	12.5	DDQNPH, SS

940.5455	471.28 (+2)	SRYPSYGL	κ-CN (f33-40)	9.44	9.25	25	SR, Y, PSY, G, L
853.3769	427.70 (+2)	PMHIRLS	β-Lg (f144-150)	11.56	9.6	57.14	PM,H, IR, L,S
925.9527	926.96 (+1)	QFLPYPY	κ-CN (f54-60)	5.36	5.36	57.14	Q,F,L, PYP, Y
1071.8986	268.98 (+4)	FGSPPGQRDL	Lactotransferrina (f289-298)	6.56	14.2	40	F, GSPPGQR, D, L
972.8655	487.44 (+2)	SSAYSRGVF	Seroalbúmina (f14-18 péptido señal; f1-4)	9.64	9.86	33.33	SSA,Y, SR, GVF
477.6737	239.84 (+2)	RGVF	Seroalbúmina (f1-4)	10.73	8.69	50	R, GVF
1114.2621	372.43 (+3)	AAHVKQVLLH	Lactotransferrina (f604-613)	10.21	13.71	60	AAH, VK, QVL, L, H
1674.3202	559.11 (+3)	DSALGFLRIPSKVDSA	Lactotransferrina (f302-317)	6.94	17.67	50	DSA, L, D, G, F, L, R, IPSK, VDSA

6. IN VITRO AND IN SILICO EVALUATION OF MULTIFUNCTIONAL PROPERTIES OF BIOACTIVE SYNTHETIC PEPTIDES IDENTIFIED IN MILK FERMENTED WITH *Lactococcus lactis* NRRL B-50571 AND NRRL B-50572

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In vitro and *in silico* evaluation of multifunctional properties of bioactive synthetic peptides identified in milk fermented with *Lactococcus lactis* NRRL B-50571 and NRRL B-50572

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ABSTRACT

The aim of this study was to investigate the multifunctional properties of specific synthetic peptides previously identified in milk fermented with *Lactococcus lactis* NRRL B-50571 and NRRL B-50572 on the inhibition of enzymes involved in hypertension (ACE), type 2 diabetes (DPP-IV) and thrombosis (thrombin). Moreover, *in vitro* gastrointestinal digestion was performed in order to know the stability of peptides and its effect on the biological activities. Also, *in silico* analysis was employed for the prediction of sequences after digestion. Results showed that peptide inhibitory activities on ACE, DPP-IV and thrombin were enhanced after digestion. After *in vitro* digestion, peptide NAVPITPTLN showed the best efficiency to inhibit DPP-IV. Likewise, NAVPITPTLN and QEPVLGPVRGPFIIV showed high thrombin inhibitory activities, and HPHPHLSFMAIPP and SLPQNIPPL presented high ACE inhibitory activities. *In vitro* and *in silico* studies indicated that these peptides are precursors of multifunctional peptide sequences. Therefore, this study suggests that the specific synthetic peptides previously identified in milk fermented with *Lactococcus lactis* NRRL B-50571 and NRRL B-50572 offer potential beneficial health effects in the management of hypertension, thrombosis and diabetes.

1. Introduction

The pathophysiology of many diseases, such as cardiovascular diseases, (CVD) involve multiple risk factors, including, hypertension and diabetes, which are associated with prothrombotic or hypercoagulability states, causing cardiovascular events (Domingueti et al, 2013 Kjeldsen, 2018; Yisireyili et al., 2016). These disorders increase three times the probability of suffering a heart attack or a cerebrovascular accident (Srivastava, 2012). Currently, therapeutic treatments have been focused on the use of pharmacological agents to reduce risk factors. However, it has been suggested that these treatments combined with the modification of lifestyle improve health status (Kirkpatrick et al., 2019). In this sense, several approaches, including the adoption of a healthy diet, are needed to reduce all possible risk factors.

It has been demonstrated that food-derived bioactive compounds are potential agents as adjuvants for cardiometabolic disorders such as hypertension and diabetes. Bioactive peptides have been recognized to be nutritionally and functionally important in health, (Martínez-Sánchez, Gabaldón-Hernández, & Montoro-García, 2020; Melini, Melini, Luziatelli, Ficca, & Ruzzi, 2019; Udenigwe & Rouvinen-watt, 2015, pp. 9303–9313). One of the most significant properties of peptides is their multifunctionallity. This feature is preferred over those that present a single biological activity, since they can exert activities simultaneously, in a pathology involving several physiological mechanisms (Aguilar-Toala et al., 2017; Lammi, Aiello, Boschin, & Arnoldi, 2019). Therefore, emphasis has been centered on the discovery of peptides that contain several functions in the same peptide sequence (Mandal et al., 2014; Pérez-Vega, Olivera-Castillo, Gómez-Ruiz, & Hernández-Ledesma, 2013; Prados, Marina, & García, 2018).

In this regard, milk proteins have been an important source of

Abbreviations: ACE, Angiotensin, converting enzyme; DPP-IV, dipeptidil peptidase IV; HHL, hipuril histidil leucine; NB-571, *Lactococcus lactis* NRRL B-50571; NB-572, *Lactococcus lactis* NRRL B-50572; L, *Lactococcus*; LAB, lactic acid bacteria; OPA, o-Phthaldialdehyde reagent; HA, hipuric acid; WSF, water-soluble fraction <3 kDa; CE, crude extract; IC_{50}, peptide content (μM) necessary to inhibit the thrombin activity by 50%.

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bioactive peptides with several biological activities (Nielsen, Beverly, Qu, & Dallas, 2017) that may be released using specific enzymes or through gastrointestinal enzymes after ingestion; however, the diversity of released peptides seems to be limited. Therefore, other strategies are preferred, such as fermentation with lactic acid bacteria (LAB) (Raveschot et al., 2018). It has been proposed that multifunctional peptides can be obtained due to a wide variety of microbial proteinases present in each strain, resulting in a variety of peptide sequences with a wide spectrum of biological activities (Raveschot et al., 2018). Hence, peptides with multiple enzyme-inhibitory activities, namely, angiotensin I converting enzyme (ACE), dipeptidyl peptidase IV (DPP-IV) and thrombin, can play a key roll in the management of hypertension, type II diabetes and thrombosis; respectively (Han, Maycock, Murray, & Boesch, 2019; Tu et al., 2017).

Milk fermentation with *Lactococcus lactis* NRRL B-50571 (NB-571) and NRRL B-50572 (NB-572) were studied in order to know their ability to release peptide fractions with ACE inhibition activity and their antihypertensive effect (Beltrán-Barrientos et al., 2018a, 2018b; Rodríguez-Figueroa, González-Córdova, Torres-Llanez, Garcia, & Vallejo-Cordoba, 2012; Rodriguez-Figueroa, Gonzalez-Cordova, Astiazaran-Garcia, Hernandez-Mendoza, & Vallejo-Cordoba, 2013). Also, thrombin inhibitory activities of water soluble fractions (<3 kDa) from these milks were reported (Rendon-Rosales et al., 2019). However, although peptide sequences present in fermented milks were identified in isolated HPLC chromatographic fractions ()Rodríguez-Figueroa et al., 2012, it is important to study the fate of these peptides under gastrointestinal conditions before bioavailability studies may be carried out; since the relationship between *in vitro* bioassays and the *in vivo* effects of bioactive peptides depends on bioavailability and stability in the digestive tract (Nongonierma & FitzGerald, 2016; Udenigwe, Abioye, Okagu, & Obeme-Nmom, 2021).

Additionally, the validation of the structure-activity relationship of specific identified sequences derived from an *in vitro* digestion model is necessary. In this sense, the evaluation of chemically synthesized peptides, is a conventional approach that combined with bioinformatics, support the discovery of novel bioactive peptides (Li-Chan, 2015). On the other hand, most natural sources of peptides are in low concentrations, making their isolation and purification difficult in sufficient quantities for carrying out studies. As a result, there has been growing interest in chemically synthesized peptides for their application in bioactivity studies (Perez Espitia et al., 2012). Therefore, the application of *in vitro* and *in silico* protocols with synthetized peptides is a prerequisite for determining peptide sequences with relevant physiological significance.

Thus, the objective of this work was to study 12 specific synthetic peptides previously identified in milk fermented with *Lactococcus lactis* NB-571 and NB-572 for their ability to present inhibitory effects on ACE, DPP-IV and thrombin enzymes after simulated gastrointestinal conditions exposure. Moreover, *in silico* digestion was performed in order to know the potential peptide sequences released that may be involved in these bioactivities.

2. Materials and methods

2.1. Reagents

Reagents used in this study were either analytical grade or of the highest purity available commercially. Thrombin from human plasma (EC: 3.4.21.5), human fibrinogen, pepsin from porcine (EC: 3.4.23.1), pancreatine from porcine, bile salts (sodium taurocholate, cholic acid, sodium deoxycholate), α-amylase (EC: 3.2.1.1), lysozyme (EC: 3.2.1.17), mucin, Angiotensin Converting Enzyme (ACE) from rabbit lung (EC: 3.4.15.1), Hippuryl histidyl leucine (HHL), DPP-IV (3.4.14.5), Gly-Pro-*p*-nitroanilide and glutathione were purchased from Sigma-Aldrich Chemicals Co. (St. Louis. MO. USA). Fluoraldehyde (OPA, o-Phthaldialdehyde reagent) was obtained from Thermo Fisher Scientific (St. Diego, CA. USA).

2.2. Synthetic peptides

Twelve synthetic peptide sequences previously identified in milk fermented with *L. lactis* NB-571 or NB-572 were used in this study (Table 1) (Rodríguez-Figueroa et al., 2012). The selection of these peptides was based on the structural similarity with peptides that offer antihypertensive effect informed in previous reports. (FitzGerald, Murray, & Walsh, 2004; Marcone, Belton, & Fitzgerald, 2017; Rodríguez-Figueroa et al., 2012). These peptides were purchased in lyophilized form and synthesized by GenScript Corporation (Piscataway, N.J. USA). For the analysis of biological activities, peptides were individually suspended in milliQ water at a final concentration of 1 mg/mL.

2.3. In vitro gastrointestinal digestion

Peptides (0.75 μL, 6.75 mg/mL) were digested using an *in vitro* gastrointestinal digestion model and the digestive solutions were prepared according to a previously reported model, specifically validated for milk through a detailed characterization of macronutrients degradation through all stages of digestion and comparing the results with physiological data from human studies (Kopf-bolanz et al., 2012). The system consisted of three stages, each simulating the mouth (1 mL of artificial saliva solution), the stomach (2 mL of gastric solution with pepsin) and the intestine (2 mL of duodenal solution with pancreatin and 1 mL of bile solution). All mixtures during the digestion process were incubated at 37 °C using an orbital shaker bath (300 rmp). A sample of milliQ water (0.75 μL) was included as a control in the digestion process. After total digestion, the digested samples were rapidly cooled and centrifuged (4696×*g*, 40 min, 4 °C). The supernatant (crude extract; CE) was recovered and peptides were isolated using centrifugal filter membranes with a cut-off of 3 kDa (Amicon Ultra-0.5, Millipore Inc. Billerica, MA, USA.) at 10 000×*g*, 15 min at 4 °C. The peptide fractions were stored frozen (−80 °C) for subsequent analysis.

2.4. Degree of hydrolysis of peptides after in vitro digestion

The degree of hydrolysis after digestion of each peptide was determined by RP-HPLC. The digested and undigested peptides were injected (20 μL) into an HPLC system (1100 series; Agilent Technologies Japan Ltd., Tokyo, Japan) consisting of a C18 column (300 Extend-C18, 4.6 × 250 mm, 5 μm particle size, Agilent technologies, Santa Clara, CA, USA)

Table 1
Synthetic peptides previously identified in milk fermented with *L. lactis* NB-571 or NB-572[a].

Peptide code	Sequence	Fragment	*L. lactis*	Molecular mass
P1	QEPVLGPVRGPFIIV	β –CN(f194-209)	NB-571 & NB-572	1,717.0
P2	YIPIQYVLS	κ-CN (f25–33)	NB-571	1,094.6
P3	HPHPHLSFMAIPP	κ-CN (f98-110)	NB-571	1,479.7
P4	YDTQAIVQ	α-La (f55–62)	NB-571	1,035.5
P5	TDDIMCVK	α-La (f86–93)	NB-572	922.4
P6	YPSYGL	β-CN (f35–40)	NB-572	698.3
P7	DVENLHLPLPLL	β-CN (f129–140)	NB-572	1,372.7
P8	SLPQNIPPL	β-CN (f69–77)	NB-571	977.6
P9	NAVPITPTLN	α_{S2} -CN (f115–124)	NB-571	1,038.6
P10	HIQKEDVPS	α_{S1} -CN (f80–88)	NB-571	1,051.5
P11	GYLAVA	Serotransferrin (f448–453)	NB-571	592.3
P12	TVQVTSTAV	κ-CN (f161–169)	NB-572	904.5

[a] As reported by Rodríguez-Figueroa et al. (2012), CN, casein; La, lactoalbumin.

with a gradient separation and a flow rate of 0.5 mL/min. The separation solvents were: solvent A (water + trifluoroacetic acid; 1000: 0.04 v/v) and solvent B (acetonitrile + trifluoroacetic acid; 1000: 0.03 v/v). The gradient program consisted of a linear increase of solvent B in A from 0.01 to 99.9 in 25 min and then return to the initial conditions. The peptides were detected at 214 nm. The degree of hydrolysis was calculated with the following equation:

Degree of hydrolysis (%) = AP-AD/AP x 100.

Where:

AD: Area of the digested peptide - area of digestion control (milliQ water)

AP: Area of undigested peptide.

2.5. ACE inhibitory activity assay

The ACE inhibitory activity of peptides was determinate according to Wu, Aluko, and Muir (2002). The enzyme and the substrate HHL were suspended in sodium metaborate solution (100 mM, 300 mM NaCl, pH 8.3), at a final concentration of 0.2 U/mL and 2.17 mM respectively. For enzymatic reaction, 50 μL of substrate were mixed with 10 μL of peptide and incubated for 10 min. Then, 10 μL of preincubated ACE solution were added to the mixture and incubated for 30 min (37 °C, 450 rpm; Eppendorf Thermomixer, Brinkmann instruments NY, USA). Afterwards, the reaction was stopped with 85 μL of 1M HCl, and the hipuric acid (HA) released in the reaction was determined using an RP-HPLC system (1260 series, Agilent Technologies, Germany) with a ZORBAX Eclipse Plus C18 column (4.6 × 100 mm, 4.8 μm; Agilent technologies, Santa Clara, CA, USA). Solvent A consisted of a mixture of water with trifluoroacetic acid (1000:0.5) and solvent B was acetonitrile with trifluoroacetic acid (1000:5). The separation of HA was performed using a gradient elution (5-60%; Solvent B in A) in 17 min and the detection of HA was obtained at 228 nm with an UV/diode array detector. The percentage of ACE inhibitory activity was calculated using the following equation.

ACE inhibition (%) = A-B/A x 100.

A = peak area of hippuric acid released of the ACE reaction with substrate; B = peak area of hippuric acid released of the ACE reaction with substrate and sample.

2.6. Thrombin inhibitory activity assay

The *in vitro* antithrombotic activity of peptides was determined using the method based on the inhibition of polymerization of fibrin catalyzed by the thrombin enzyme according to Yang, Wang, and Xu (2007). Briefly 140 μL of fibrinogen (0.1% w/v, dissolved in 50 mM TRIS-HCl with 0.12 mM NaCl, pH 7.2) and 40 μL of peptide were mixed and the absorbance was taken at 405 nm after 10 min of incubation at 37 °C. Thereafter, 10 μL of thrombin (12 U/mL) were added to the mixture and incubated once more for 10 min and finally the absorbance was recorded. Thrombin inhibition was calculated with the following equation.

$$\text{Thrombin inhibition} = {}_{[}[(A\text{-}B)\text{-}(C\text{-}D)]/(A\text{-}B)_{]} \text{ X } 100$$

Where A = positive control (substrate + enzyme + buffer), B = negative control (substrate + buffer + buffer), C = test sample (substrate + enzyme + sample), and D = test sample blank (substrate + buffer + sample).

2.7. DPP-IV inhibitory activity assay

The synthetic peptides as well as the digested peptides were tested for their inhibition effect against DPP-IV activity using the chromogenic substrate Gly-Pro-*p*-nitroanilide as previously reported by Lacroix & Li-Chan, 2014. Briefly, 25 μL of each digested or undigested peptides were pre-incubated with 25 μL of substrate (1.59 mM in 100 mM Tris-HCl buffer, pH 8.0) at 37 °C during 10 min. Afterwards 50 μL of DPP-IV (0.02 U/mL in100 mM Tris-HCl buffer, pH 8.0) were added and the reaction was incubated for 60 min at 37 °C. Then, the reaction was stopped by adding 100 μL of sodium acetate buffer (1M, pH 4.0) and the absorbance of the final reaction was measured at 405 nm. The percentage of DPP-IV inhibition was calculated with the following equation.

$$\text{DPP-IV inhibition} = {}_{[}[(A\text{-}AB)\text{-}(S\text{-}SB)]/(A\text{-}AB)_{]} \text{ X } 100$$

Where A = positive control (substrate + enzyme + buffer), AB = negative control (substrate + buffer + buffer), S = test sample (substrate + enzyme + sample), and SB = test sample blank (substrate + buffer + sample).

For ACE, thrombin and DPP-IV inhibition activities, results were also expressed as peptide content (μM) necessary to inhibit enzyme activity by 50% (IC_{50}). Peptide content after the digestion process was estimated by the quantification of primary amino nitrogen in fractions (≤3 kDa) according to the method described by Wang et al. (2008) using the OPA method. Glutathione (0-250 μg/mL) was used as a standard. Concentration in μM was calculated by using peptides molecular weight.

2.8. Prediction of amino acid sequences using in silico digestion of specific peptides

In silico digestion analysis was performed using the Peptide Cutter Proteomic at from Bioinformatic Resource Portal ExPASy (www.expasy.org) from the Swiss Institute of Bioinformatics (SIB). Peptides sequences were individually digested with gastrointestinal enzymes present in the *in vitro* model digestion, namely pepsin, trypsin, chymotrypsin and elastase (Nongonierma & Fitzgerald, 2014). The number map, cleavage sites and the final resulting peptide sequences were obtained. Additionally, theoretical properties of peptides such as hydrophobicity, charge, and molecular weight were obtained using PepDraw (Tulane University, 2011). Finally, the database BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) (Minkiewicz, Iwaniak, & Darewicz, 2019) was used in order to know the possible peptide bioactivities released after digestion. Moreover, the Peptide Ranker tool (http://distilldeep.ucd.ie/PeptideRanker/) was used in order to know the probability that the final sequences would be bioactive.

2.9. Statistical analysis

The results presented in this study were obtained from two independent experiments and each analysis was performed by triplicate. The data were analyzed using NCSS 2007 software. For the statistical analysis, one-way ANOVA was performed for the comparison between the different peptides; in case of significant differences, the means were compared using Tukey-Kramer test. The t-paired test was used to analyze peptide differences before and after *in vitro* digestion. For all analyzes, differences were considered significative when $p < 0.05$. The data are expressed as the mean ± standard deviation (SD).

3. Results and discussion

3.1. Degree of hydrolysis of specific peptides after in vitro digestion

A predominant challenge for peptide biological activities is their passage through the gastrointestinal system after oral consumption. Since, the function of the peptides depends on their structure (size and amino acid sequence) and physicochemical properties (charge, hydrophobicity), they can be affected by gastrointestinal conditions such as proteolytic enzymes and absorption though enterocytes (Sun, Acquah, Aluko, & Udenigwe, 2020; Wang & Li, 2018). Therefore, the degree of hydrolysis of peptides after being exposed to gastrointestinal proteolytic enzymes was evaluated (Fig. 1). All peptides were partially hydrolyzed, particularly, peptides 2 and 3 were the most hydrolyzed (>80%, $p <$

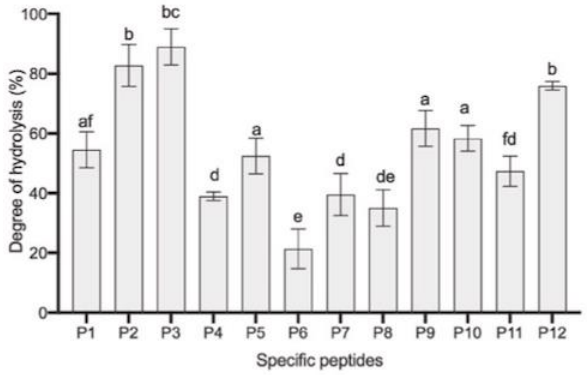

Fig. 1. Degree of hydrolysis (%) of specific peptides (P1-12) after *in vitro* digestion. Bars represent the mean (n = 3) ± standard deviation (SD). Letters indicate significant differences ($p < 0.05$) between specific peptides.

0.05) while peptides 6 and 8 were more resistant to degradation (<30% $p < 0.05$). The rest of the peptides were hydrolyzed within a range of 40–60%. It is important to consider that gastrointestinal enzymes (*in vivo* and *in vitro*) might not completely hydrolyze at their peptide cleavage site (miss cleavage). The above depends on factors such as accessibility of peptides by the target enzyme on the cleavage site, and the structural conformation of peptides that will impact on the hydrolysis degree (Han et al., 2019). It has been reported that peptides released after gastrointestinal digestion may improve their biological activity (Villadóniga, María, & Cantera, 2019). Moreover, small peptides (dipeptides and tripeptides) can be easily and efficiently be transported in the apical membrane or the enterocytes, be absorbed and exert their activity (Adam, Mohd, & Gan, 2019; Xu, Hong, Wu, & Yan, 2019).

3.2. ACE inhibitory activity

ACE is a key element in the regulation of blood pressure, which catalyzes the production of angiotensin II from angiotensin I, resulting in the contraction of arteries. Moreover, ACE degrades the bradykinin, a vasodilator peptide, causing high blood pressure. Therefore, the inhibition of ACE is an effective treatment of hypertension (Guo et al., 2019; Han, Maycock, Murray & Boesch et al., 2019; Moslehishad et al., 2013). In this regard, peptides were investigated for their ability to inhibit ACE. Fig. 2A shows the percentages of ACE inhibition of peptides before and after their exposure to gastrointestinal conditions. From twelve intact peptides, half of them showed ACE inhibitory activity (P2, P3, P5, P6, P11, P12) above of 70%. In order to establish the inhibition efficiency of peptides, the dose-response relationship was investigated to determine the IC_{50}. Table 2 summarizes the IC_{50} values of ACE inhibition. Among the six intact peptides that showed ACE inhibitory activity (%), peptide 2 (YIPIQYVLS) exhibited the best inhibitory activity (low IC_{50} value) ($p < 0.05$) of all before gastrointestinal digestion.

After *in vitro* gastrointestinal conditions, intact peptides that did not show ACE inhibitory activity before digestion, increased their activity significantly ($p < 0.05$) by more than 30%, with peptide 3 and peptide 8 showing the highest inhibition values (>50%). Moreover, both digested peptides were more efficient because it required less peptide concentration to inhibit 50% of ACE (Table 2). Likewise, peptide 2 also showed a low IC_{50} value (Table 2), despite the fact that the inhibition percentage was ≈50%. The increase of inhibitory activity may be due to the sequence composition released after digestion. In general, it is accepted that ACE inhibitory peptides depend on the three amino acids located in the C-terminal. For this reason, amino acids such as P, Y, F, W and L are important for the inhibition efficiency (Jiang, Tian, Brodkorb, & Huo, 2010; Manoharan, Shuib, & Abdullah, 2017). Note that for the *in silico* study, the released fragments (Table 3) for peptide 8 were, SL and PQNIPPL and for peptide 3 were HPHPL, SF, MA and IPP. Therefore, since these peptides contain P and L in their C-terminal sequence, their effectiveness could be associated with these amino acids in C-terminal positions. Furthermore, the widely reported IPP sequence with strong ACE inhibition was also a structural part of these peptides with high inhibition efficiency (Chakrabarti, Liao, Davidge, & Wu, 2017; Yamaguchi, Kawaguchi, & Yamamoto, 2009). Finally, the findings reported in the present study strengthens the fact that the antihypertensive effect of milk fermented with either *L. lactis* NB-571 or NB-572 (Beltrán-Barrientos, Hernández-Mendoza, et al., 2018; Rodriguez-Figueroa et al., 2013) may be due to the action of these ACE inhibitory peptides, particularly those that maintained or increased their activity after digestion. This indicates that, the peptide sequences released during fermentation are precursors for the subsequent release of peptide sequences, with high ACE inhibitory activity, during gastrointestinal digestion.

3.3. Thrombin inhibitory activity

Thrombin is an enzyme that plays an important role in hemostasis and coagulation. In pathological conditions, such as hypercoagulability or prethrombotic state, inhibition of the coagulation cascade by blocking thrombin is effective in preventing thrombosis and CVD (Negrier, Shima, & Hoffman, 2019). Therefore, these specific peptides were investigated for their thrombin inhibitory activity. As shown in Fig. 2B, nine peptides showed low inhibition activities (≈8-17%), while the remaining three peptides showed no activity (P4, P1, P12). Interestingly, the peptide QEPVLGPVRGPFIIV studied here showed low activity, compared to the study published by Rojas-Ronquillo et al. (2012). In that study, authors identified a similar sequence (YQEPVLGPVRGPFPIIV) in a chromatographic fraction derived from casein hydrolysates, with thrombin inhibitory activity. This may suggest that tyrosine at the C-terminal residue may play a key role in thrombin inhibition.

After gastrointestinal digestion, the inhibitory activities of ten peptides were significantly ($p < 0.05$) enhanced up to ten-fold. Among these peptides, seven sequences (P1, P5, P6, P7, P8, P10, P11) completely inhibited thrombin activity (>90%). The IC_{50} values shows that P9, P1, P7 and P10 were the more efficient for thrombin inhibition (Table 2). NA, PITPTL, QEPV, GPV, GPF, IIV, IQK, EDV, PS, DV, EN and PLP were the sequences released after *in silico* digestion of P9, P1, P7 and P10; therefore, these fragments might be responsible for thrombin inhibition. It is important to emphasize that these fragments have not been reported as thrombin inhibitor peptides. In this sense, this study provides an insight into the discovery of novel sequences with potential antithrombotic effect.

There are few references describing the molecular mechanisms of thrombin inhibition by peptides, it has been reported that charged amino acids, particularly negatively charged, in the C-terminal, play an important role in the binding at the active site or exosites of thrombin (Riester et al., 2005; Tu et al., 2017; Zhang, 2016). However, some authors indicate that thrombin inhibition by peptides seems to be more complex. Molecular docking studies reveal that inhibition involves several interactions such as, attractive charges, hydrogen bonds, hydrophobic alkyls and salt bridges (Chen & Huang, 2020). Therefore, for thrombin inhibition, peptides require both, negatively charged as well as hydrophobic amino acids in their sequences. In this study, peptides with negative charge and uncharged, seem to be associated with higher inhibition activities. Nevertheless, to clarify this association, molecular docking studies are required to establish the inhibition mechanisms of peptides that were here studied.

On other hand, it was reported that the water soluble fraction (WSF, <3 kDa) derived from milk fermented with *L. lactis* NB-571 or NB-572 showed thrombin inhibitory activity, and this activity significantly improved after an *in vitro* gastrointestinal process (Rendon-Rosales et al.,

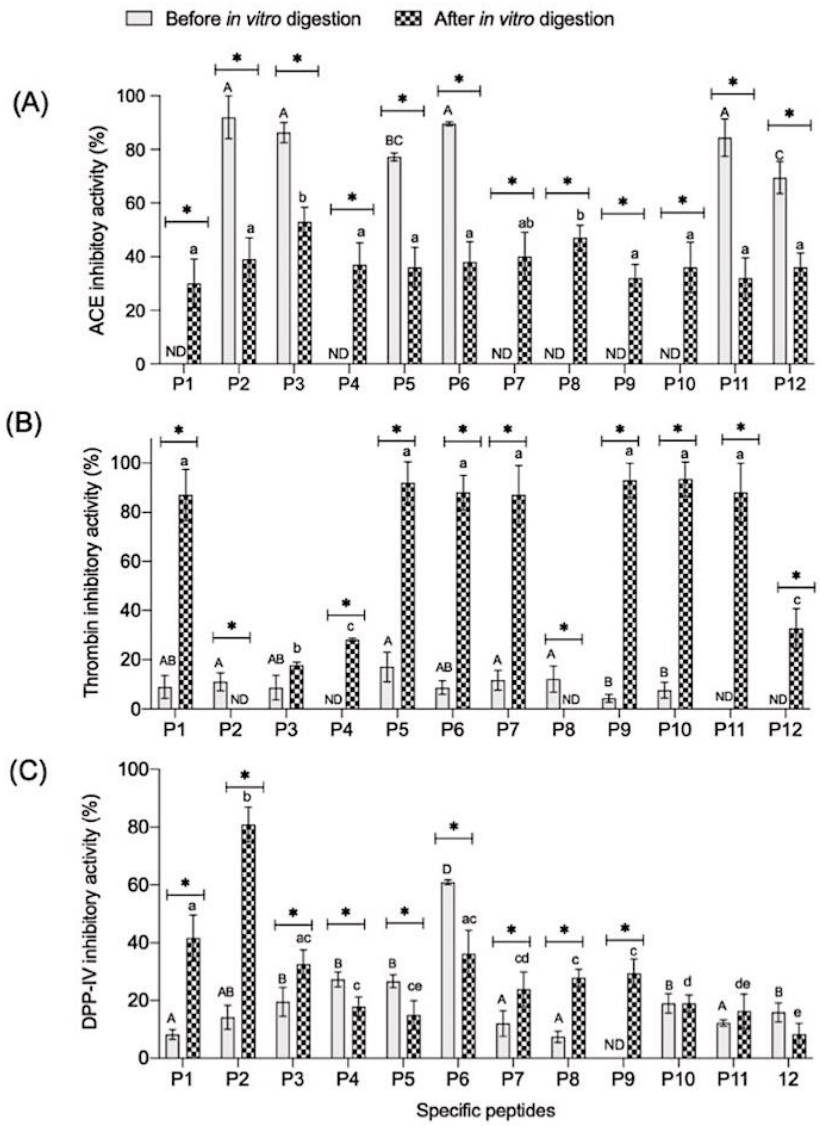

Fig. 2. (A) ACE inhibitory activity (%), **(B)** Thrombin inhibitory activity (%) and **(C)** DPP-IV inhibitory activity (%) of specific peptides (P1-12), before and after *in vitro* digestion. Bars represent the mean (n = 3) ± standard deviation (SD). Capital letters indicate significant differences ($p < 0.05$) between peptides before digestion. Lowercase letters indicate significant differences ($p < 0.05$) between peptides after digestion. Asterisk (*) indicates significant differences ($p < 0.05$) before and after *in vitro* digestion for each peptide.

2019). Therefore, the findings reported in this study may support the improvement of thrombin inhibitory activity of WSF after the digestion process.

3.4. DPP-IV inhibitory activity

Incretins (Glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide) are the main hormones that maintain glucose homeostasis through the stimulating secretion of pancreatic insulin. Furthermore, incretins retard gastric emptying and modulate appetite. However, these hormones are easily degraded by DPP-IV (Ayyash, Al-Dhaheri, Mahadin, Kizhakkayil, & Abushelaibi, 2018; Han et al., 2019; Müller et al., 2019). Therefore, food-derived peptides with inhibitory action on DPP-IV are a promising approach in the management of diabetes. Fig. 2C shows the inhibitory activities of synthetic peptides before and after gastrointestinal exposure. Most of the peptides before gastrointestinal exposure presented from low to moderate inhibitory activity against DPP-IV (>8%). Peptide 6 (YPSYGL) showed the highest inhibition activity with 62-%, YPSYGL has only been reported as an ACE inhibitory peptide, but not as a DPP-IV inhibitor. Thus, to the best of our knowledge, this is the first report showing that YPSYGL has dual ACE and DPP-IV inhibitory activities.

After gastrointestinal digestion, peptides P1, P2, P8 and P9 improved their inhibitory activities ($p < 0.05$) and IC_{50} values decreased significantly ($p < 0.05$) (Table 2). P8 and P9 showed the lowest values with 368.24 and 298.47 μM, respectively (Table 2). In general, it has been reported that peptides with DPP-IV inhibitory activity, contain proline in either its N- or C-terminal. Likewise, in position two, various hydrophobic, charged and neutral amino acids could enhance binding with the catalytic site of the enzyme (Lacroix & Li-Chan, 2012, 2014; Liu, Cheng, & Wu, 2019). In this study, several of the fragments released by *in silico* digestion of peptides were previously reported as DPP-IV inhibitors (Nongonierma et al., 2018; Nongonierma & FitzGerald, 2015). These fragments are P1 (GPV, GPF), P2 (IPIQ), P3 (SF, MA), P7 (PLP), P8 (SL, PQNIPPL), P9 (NA, PITPTL) and P10 (PS). In fact, these fragments were found for those digested peptide sequences that showed high

Table 2
ACE, thrombin and DPP-IV inhibitory activities (IC_{50}, μM) of specific peptides (1-12), before and after *in vitro* digestion.

Peptide	ACE inhibitory activity IC_{50} (μM)		Thrombin inhibitory activity IC_{50} (μM)		DPP-IV inhibitory activity IC_{50} (μM)	
	Before *in vitro* digestion	After *in vitro* digestion	Before *in vitro* digestion	After *in vitro* digestion	Before *in vitro* digestion	After *in vitro* digestion
P1	n.d.	>372.74	>582.41	151.42 ± 8.23 [b]	>582.41	375.65 ± 12.35 [b]
P2	60.29 ± 5.1[a]	388.27 ± 32.29 a*	>913.57	n.d	>913.57	433.94 ± 32.29 [c]
P3	82.78 ± 12.3[b]	206.12 ± 43.99 b*	>675.81	>489.28	>675.81	>489.28
P4	n.d.	>705.93	n.d.	>705.93	>965.717045	>705.93
P5	60.71 ± 6.1[a]	>715.52	>1084.12	374.02 ± 38.32 [e]	>1084.12	>715.52
P6	719.60 ± 55.6[c]	>1632.54	>1432.04	959.47 ± 40.50 [g]	1231.99 ± 17.50	>1632.54
P7	n.d.	>371.53	>728.49	185.76 ± 15.75 [c]	>728.49	>371.53
P8	n.d.	276.19 ± 43.39 [b]	>1022.91	n.d.	>1022.91	368.24 ± 28.93 [b]
P9	n.d.	>192.57	>962.83	86.65 ± 13.61[a]	n.d.	298.47 ± 13.61[a]
P10	n.d.	>551.59	>951.92	264.85 ± 15.56 [d]	>951.92	>551.59
P11	934.49 ± 32.2[d]	>1502.62	n.d.	785.07 ± 83.56 [f]	>1688.33	>1502.62
P12	681.03 ± 23.4[c]	>884.47	n.d.	>884.46	>1105.58	>884.47

n.d. No activity was detected with the specific digested and undigested peptide.
(>) Maximal concentration of peptide used in the assays or total concentration of peptide obtained after *in vitro* digestion.
The values are expressed as mean (n = 3) ± standard deviation (SD).
* Significant differences before and after *in vitro* digestion.
Letters indicate significant differences between peptides for the same condition (digested or undigested peptides).

Table 3
In silico gastrointestinal digestion of specific bioactive peptides and potential activity of released fragments after *in silico* digestion.

Synthetic peptide	Enzymes	Fragments released by gastric enzyme*	Fragments releases by intestinal enzymes**	Bioactivities[3]
QEPVLGPVRGPFIIV	Elastase, chymotrypsin LE[1], trypsin, chymotrypsin HE[2]-LE	QEPVLGPVRGPFIIV	QEPV, GPV, GPF, IIV, L, R	ACE inhibitor (GPV) DPP-IV inhibitor (GPV)
YIPIQYVLS	Pepsin (pH > 2), chymotrypsin HE-LE, elastase,	YIPIQ, Y, V, L, S	YPIQ, Y,Y, V, L, S	n.d.
HPHPHLSFMAIPP	Pepsin (pH > 2), chymotrypsin HE-LE Elastase	HPHPHL, SF, MAIPP	HPHPH, L, SF, M, A, IPP	ACE inhibitor (IPP) Anti-inflammatory (IPP)
YDTQAIVQ	Pepsin (pH > 2), Chymotrypsin HE-LE, Elastase	Y, DTQAIVQ	Y, DTQA, IV, Q	Glucose uptake stimulating peptide (IV)
TDDIMCVK	Chymotrypsin LE, elastase	TDDIMCVK	TDDIM, CV, K	n.d
YPSYGL	Pepsin (pH > 2)	Y, PSY, G, L	Y, PSY, G, L	ACE inhibitor (PSY)
DVENLHLPLPLL	Pepsin (pH > 2), Elastase, Chymotrypsin LE,	DVEN, L, HL, PLP, LL	DV, EN, L, H, PLP, L,L,L,L	ACE inhibitor (PLP)
SLPQNIPPL	Pepsin (pH > 2)	SL, PQNIPPL	SL, PQNIPPL	DPP-IV inhibitors
NAVPITPTLN	Pepsin (pH > 2) Elastase, chymotrypsin LE,	N, NAVPITPTL	N, NA, V, PITPTL	DPP-IV inhibitor (NA)
HIQKEDVPS	Chymotrypsin LE, trypsin, elastase,	HIQKEDVPS	H, QK, EDV, PS	DPP-IV inhibitors (PS)
GYLAVA	Pepsin (pH > 2), chymotrypsin HE-LE	G, Y, L, AVA	G, Y, L, A, V, A	n.d.
TVQVTSTAV	Elastase,	TVQVTSTAV	TV, QV, TSTA, V	DPP-IV inhibitors (TV,QV) Antioxidative (TSTA)

[1]Low specificity, [2]High specificity Chymotrypsin, Pepsin was used in gastric digestion, **Trypsin, chymotrypsin and elastase were used in intestinal digestion, Activities were provided by BIOPEP Database, n.d. No data reported for specific peptide.

DPP-IV inhibitory activities. These fragments contain proline and hydrophobic amino acids in their sequence, suggesting that they may be responsible for the inhibition of DPP-IV.

3.5. Prediction of peptide sequences released after in vitro digestion

In silico digestion was performed in order to observe the impact of gastrointestinal enzymes on the structure of these peptides. Consequently, the gastrointestinal enzymes presents in the *in vitro* model (pepsin, trypsin, chymotrypsin and elastase) were considered. Table 3 summarizes peptides and the possible fragments released after their exposure to *in vitro* gastrointestinal digestion. Moreover, Table 3 enlisted the enzymes responsible for hydrolysis and the cleavage number and position. In general, peptide sequences were mostly affected by pepsin, chymotrypsin and elastase, since all these enzymes hydrolyzed almost all peptides and also cleaved in several peptide bonds for the same peptide. On the contrary, peptides were less affected by trypsin with only one proteolytic cut in two peptides (P1 and P10). Three peptides, YIPIQYVLS (P2) DVENLHLPLPLL (P7), and GYLAVA (P11) were highly susceptible to hydrolysis, of which, GYLAVA was completely hydrolyzed to their amino acid residues. These peptides were hydrolyzed by most of the enzymes or by a greater number of cleavages by the same enzyme. Meanwhile, SLPQNIPPL (P8) and TDDIMCVK (P5) sequences were the least affected in only one proteolytic cleavage. This can be corroborated with the determination of the degree of hydrolysis (Fig. 1), where P2 exhibited a higher degree of hydrolysis while P8 presented less degradation. In this study, the bioactivity profile of the sequences released after digestion was also analyzed using the BIOPEP database. The findings show that some fragments are reported to present ACE (QEV, IPP, PSY, PLP) and DPP-IV (GVP, SF, MA, PQNIPPL, NA, PIPIPTL, PS, TV) inhibitory activities. Thus, this may confirm the inhibitory activities of peptides investigated in this study, specifically after simulated gastrointestinal digestion. Additionally, according to the BIOPEP database, some fragments found by *in silico* analysis in this study have also been reported with other bioactivities. For example, TSTA (Cheng, Chen, & Xiong, 2010) showed antioxidant activity and IPP peptide showed anti-inflammatory activity in 3T3 cells (Chakrabarti et al., 2017), which supports the multifunctional activity of peptides.

Additionally, the Peptide Ranker tool was used as a probability (0-1) predictor of bioactivity of peptide fragments released from *in silico*

digestion. In this regard, the Peptide Ranker is a database, which provides a prediction based on the specific structural characteristics that make peptides bioactive. For that, a threshold of 0.5 was established, where <0.5 and >0.5 indicates lower and higher probability of bioactivity, respectively (Mooney, Haslam, Pollastri, & Shields, 2012). From all the fragments, GPF (0.98), SF (0.94), PLP (0.86), PQNIPPL (0.80), IPP (0.76), and MA (0.69) showed the highest probabilities to exert any biological activity. Indeed, two of these fragments (IPP and PLP) showed ACE inhibitory activities and three (SF, MA, PQNIPPL) presented DPP-IV inhibitory activities by the BIOPEP database.

In summary, our results support the fact that the peptides investigated demonstrate their role as precursors of fragments with multifunctional biological activities. Most of these peptides exerted their activity for inhibiting ACE, thrombin and DPP-IV enzymes, after gastrointestinal digestion.

4. Conclusions

The results suggest that the synthetic bioactive peptides identified in milk fermented with the *L. lactis* NB-571 or NB-572 were capable of releasing fragments with multifunctional properties (ACE, thrombin and DPP-IV inhibition) after their exposure to gastrointestinal digestion. Therefore, these fragments may contribute to the management of hypertension, diabetes and thrombosis. Nevertheless, *in vivo* studies are needed in order to confirm the multifunctional properties of these peptides and study their bioavailability. Also, to clarify the mechanisms involved, it is necessary to perform molecular docking and carry out studies on inhibition modes.

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CRediT authorship contribution statement

Miguel Á. Rendón-Rosales: Writing – original draft, Investigation. **María J. Torres-Llanez:** Software, Methodology. **Miguel A. Mazorra-Manzano:** Methodology, Formal analysis. **Aarón F. González-Córdova:** Supervision, Resources. **Adrián Hernández-Mendoza:** Validation, Visualization. **Belinda Vallejo-Cordoba:** Conceptualization, Writing – review & editing, Project administration.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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7. RESULTADOS Y DISCUSIÓN

La aterosclerosis es un proceso que involucra múltiples factores de riesgo. No obstante, de todos ellos, la dislipidemia es la que tiene mayor influencia en su desarrollo. Por lo tanto, el manejo de la dislipidemia como la hipercolesterolemia es fundamental para reducir el riesgo cardiovascular. Por lo que, se han utilizado varios enfoques para mejorar los niveles de lípidos. (Virani *et al*., 2020). En este trabajo se planteó la evaluación de leches fermentadas con cepas específicas de *L. lactis* como potencial coadyuvante en el tratamiento de la hipercolesterolemia mediante estudios *in vitro*, *ex vivo*, *in vivo* e *in silico*. Previamente, de cinco cepas de *L. lactis* evaluadas por su capacidad de fermentar leche se observó que tres de ellas (FM-571, FM-572 y FM-600) mostraron potencial efecto hipocolesterolémico. Este potencial fue dado por fracciones que mostraron *in vitro* capacidad para inhibir la formación de micelas de colesterol y unirse a sales biliares, tales como el taurocolato de sodio y ácido cólico. Estas actividades biológicas mejoraron después de que las leches fermentadas fueran sometidas a condiciones gastrointestinales simuladas. El tiempo de fermentación también fue evaluado, sin embargo, los mejores resultados se obtuvieron a las 48 h de fermentación (Rendón Rosales et al., 2019).

En el presente trabajo, las leches fermentadas FM-571, FM-572 y FM-600 fueron seleccionadas para evaluar su efecto hipocolesterolémuco en un modelo murino con hipercolesterolemia inducida durante siete semanas. Adicionalmente, una leche no fermentada (LNF) fue incluida dentro de los tratamientos. Los primeros resultados confirmaron el efecto hipocolesterolémico de estas leches fermentadas mostrando una diferencia marcada entre las leches fermentada y la no fermentada. Específicamente, el consumo de FM-572 y FM-600 en ratas redujo significativamente los niveles de colesterol total en un rango de 13 a 20%, y colesterol no-HDL (no-HDL-C: C-LDL+C-VLDL) en un rango de 20 a 23% ($p < 0.05$), en comparación con el grupo control (solo dieta alta en colesterol) y con el grupo tratado con LNF. Estos efectos se reflejaron en un menor índice aterogénico y riesgo cardiovascular ($p < 0.05$). La reducción de eventos cardiovasculares (*i.e.*, infartos, isquemia miocárdica) por la reducción de colesterol ya ha sido muy establecido en estudios sistemáticos y de metaanálisis. De hecho, se ha reportado que, en humanos, la reducción de 1.8 mmol/ de C-LDL reduce hasta un 17% de infartos y un 60% de enfermedad isquémica (Law *et al*., 2003).

Adicionalmente, el consumo de FM-600 redujo la acumulación de lípidos totales y colesterol en hígado. Debido a estos resultados, FM-600, aunado a su efecto reductor del colesterol, también podría ser empleada como coadyuvante en el tratamiento de la enfermedad hepática grasa no alcohólica (Schleicher *et al.,* 2014). Para observar los efectos de excreción de lípidos debido a la inhibición de las micelas de colesterol y unión a sales biliares por fracciones peptídicas, se cuantificó el colesterol y las sales biliares en heces. Interesantemente, no se observó una mayor excreción fecal de colesterol y sales biliares por el consumo de las leches fermentadas con *L. lactis*. En cambio, si se observó ligeramente una mayor excreción de lípidos totales. Lo anterior podría sugerir que la inhibición de micelas de colesterol y la unión de sales biliares por péptidos no serían los mecanismos asociados al efecto. No obstante, se ha reportado que péptidos hipocolesterolémicos con dichos mecanismos no siempre se reflejan en una mayor excreción de estos compuestos, ya que es difícil de observar en modelos *in vivo* con una dieta alta en colesterol y sales biliares (Lapphanichayakool *et al*., 2017). Lo anterior también puede explicarse por el hecho de que la microbiota se encuentra implicada en el metabolismo de colesterol y sales biliares (Witkowski *et al*., 2020).

En este sentido, en el sistema gastrointestinal los lípidos (v.g. colesterol y sales biliares) no absorbidos en el intestino delgado pueden ser metabolizados por la microbiota a nivel intestino delgado o bien en el colon. Por ejemplo, el grupo de *Eubacterium coprostanologenes* se metaboliza el colesterol a coprostanol. El coprostanol es un esterol no absorbible que luego es excretado por las heces. (Li et al., 1998*)*. Esta eliminación pudiera explicar el aumento de la excreción de lípidos y no de colesterol observados en este estudio. Además, el metabolismo de las sales biliares por la microbiota podría ser un factor que impacte en la concentración de las sales biliares en heces (Schoeler y Caesar, 2019). A este respecto, la modulación de la microbiota intestinal por el consumo de leches fermentadas también pudiera explicar la regulación de lípidos observada en este estudio.

A este respecto, para el estudiar la participación de la microbiota intestinal en la regulación de lípidos se analizó la composición de la microbiota intestinal en la mucosa colonica y heces mediante la secuenciación de la región V3-V4 del gen del ARNr 16S. Primeramente, los hallazgos mostraron que la diversidad de la microbiota fue distinta entre ambas muestras ($p < 0.05$) lo cual

concuerda con informes previos (Durbán et al., 2011). Por otro lado, la hipercolesterolemia inducida por dieta alta en colesterol modificó la estructura de la microbiota tanto para la microbiota fecal y mucosal. Específicamente, la abundancia de *Lactobacillaceae* y *Ruminococcaceae* disminuyó, mientras que la abundancia de *Bacteroidaceae*, *Tannerellaceae* y el grupo de *Eubacterium coprostanoligenes* aumentó.

El consumo de leches fermentadas modificó la estructura de la microbiota fecal pero no de la microbiota asociada a la mucosal tal como lo indica el análisis de beta diversidad. Lo anterior sugiere que las leches fermentadas presentan capacidad para modular la microbiota luminal, la cual esta mejor representada en las heces. Se ha descrito que la microbiota asociada a la mucosa es más estable con respecto a la luminal. Wu et al., 2020 demostró que este tipo de microbiota presenta "función digestiva", de hecho, la microbiota luminal colonica presentó mejor capacidad en el metabolismo de lípidos que la microbiota mucosal colonica. En las heces, las leches fermentadas mejoraron la abundancia de *Lachnospiraceae, Ruminococcaceae y Lactobacillaceae*. Para establecer la asociación entre los taxones identificados y el perfil de lípidos se realizó un análisis de correlación entre dichas variables. Siete familias en heces y tres en mucosa se correlacionaron negativamente con el colesterol total. Para C-LDL, cinco familias en heces y una familia en mucosa se correlacionaron positivamente. Lo anterior, podría corroborar que el mejor efecto modulador y efecto en el metabolismo de lípidos de las leches es a nivel luminal lo cual está presentando por las heces.

Los análisis de correlación mostraron una asociación negativa ($p<0.05$) entre *Ruminococcaceae, Lactobacillaceae, Lachnospiraceae, Oscillospiraceae, Anaerovoracaceae, Erysipelotrichaceae* y los niveles de colesterol total y non-HDL y positiva ($p < 0.05$) con C-HDL. Asimismo, El grupo de *Eubacterium coprostanoligenes* se asoció negativamente con C-LDL y colesterol fecal, esta asociación podría indicar que *Eubacterium coprostanoligenes* disminuye los niveles de colesterol en el intestino con la subsecuente producción de coprostanol (Ren *et al*., 1996; Juste y Gérard, 2021), disminuyendo así, la absorción de colesterol junto con una mayor excreción de coprostanol y niveles bajos de colesterol sanguíneo. Lo anterior fue sustentado por el estudio de Li *et al*. (1998), en el cual indicaron que la administración de estas bacterias se asoció significativamente con menores niveles de colesterol. En este sentido, se ha reconocido que *Eubacterium*

coprostanoligenes puede utilizarse para el manejo de la hipercolesterolemia.

Por otro lado, el consumo de las leches fermentadas también aumentó los niveles de AGCC, ya que la hipercolesterolemia redujo marcadamente los niveles de AGCC. Se observó un incremento de propionato con el consumo de todas las leches fermentadas. Acetato incrementó con FM-572 y FM-600, mientras que el butirato incrementó con el consumo de FM-571. El análisis de correlación permitió asociar significativamente algunos grupos microbianos con los niveles de AGCC. Por ejemplo, acetato se asoció positivamente con *Oscillospiraceae y Prevotellaceae*. Propionato se asoció positivamente con *Maribaculaceae, Bacteroidaceae y Oscillospiraceae*, mientras que butirato se asoció positivamente con *Lactobacillaceae, Ruminococcaceae, Christensenellaceae y Oscillospiraceae*. Por otra parte, de todos los ACGG, solo butirato se asoció de manera significativa con los niveles de lípidos. De manera particular, butirato se correlacionó negativamente ($p<0.05$) con colesterol total, no-HDL y triglicéridos, y de manera positiva con C-HDL ($p<0.05$). Estas observaciones sugieren, por lo tanto, que el butirato producido por la administración de las leches fermentadas es el efector de la regulación de los niveles de colesterol.

Coincidentemente, los niveles de butirato se asociaron positivamente con algunos grupos bacterianos productores de butirato (*v.g Ruminococcaceae*), y que también se asociaron negativamente con los niveles de lípidos. Lo anterior, podría sugerir que el efecto hipocolesterolémico este asociado con el aumento de estas familias. Como se ha descrito anteriormente, el butirato puede disminuir los niveles de C-LDL por varios mecanismos. Principalmente a través de la supresión de SREBP2 e inhibición de HMC-CoA reductasa, y en el aumento del eflujo de colesterol (Chambers *et al*., 2018, Hara *et al*., 1999, Alvaro *et al*., 2008). La asociación positiva con C-HDL puede explicarse por un posible aumento en la expresión de ABCA1 inducido por butirato. Los transportadores ABCG1 desempeñan un papel importante en el eflujo de colesterol de los tejidos periféricos a HDL. La expresión de ABCA1 promueve la maduración de HDL y un mayor eflujo de colesterol (Du *et al*., 2020). Por otro lado, la regulación de colesterol también podría estar mediada por la adsorción de esta molécula a la superficie celular de algunas bacterias (Pereira *et al*., 2002), por ejemplo, *Lactobacillus*. En este estudio la abundancia de *Lactobacillus* mejoró con la ingesta de las leches fermentadas, por lo que este aumento podría tener un impacto en la regulación de colesterol.

Las leches fermentadas son ricas en compuestos bioactivos, tales como los péptidos derivados por la proteólisis bacteriana. A este respecto, el presente estudio evaluó el potencial efecto hipocolesterolémico de péptidos aislados de leches fermentadas después de ser sometidas a una digestión gastrointestinal simulada. Primeramente, los resultados mostraron que las fracciones peptídicas pre-purificadas por cromatografía de exclusión por tamaño mostraron capacidad para inhibir la formación de micelas de colesterol. No obstante, la mejor actividad inhibitoria (%) se encontró en la fracción peptídica (Fracción 3) en un rango de peso molecular estimado de 0.29 a 0.62 kDa proveniente de FM-572 y de FM-600, pero con la mejor eficiencia de inhibición por la fracción peptídica proveniente de FM-572. La subsecuente purificación de péptidos por RP-HPLC indicó que las cuatro fracciones colectadas mostraron actividades inhibitorias. Sin embargo, las fracciones provenientes de la Fracción 3 (0.29-0.62 kDa) de FM-572 fueron las más eficientes para inhibir la solubilidad micelar del colesterol. Lo anterior fue consistente con estudios previos, los cuales indicaron que la mayoría de los péptidos hipocolesterolémicos (inhibidores de la solubilidad micelar) presentaron un peso molecular en un rango de 300-800 Da para péptidos de soya (Zhong *et al*., 2007) o 486.24- 659.35 Da (Jian et al., 2020) de péptidos derivados de caseína. Se ha descrito, que independientemente del peso molecular, una de las propiedades que mejor describe a los péptidos hipocolesterolémicos son la hidrofobicidad y la presencia de aminoácidos aromáticos (Zhang *et al*., 2012).

Para continuar explorando el potencial hipocolesterolémico de los péptidos, se analizó la actividad inhibitoria sobre HMG-CoA reductasa. Para ello, las leches fermentadas expuestas a digestión gastrointestinal se sometieron a un proceso de absorción intestinal basado en un modelo *ex vivo* de intestino de rata. Los péptidos fueron absorbidos en un rango de 3.23 a 7.39%. Los péptidos derivados de la leche sin fermentar y de FM-572 fueron mayormente absorbidos en comparación con FM-600. Se ha descrito que el grado de absorción de péptidos depende del tamaño de los péptidos y de las características fisicoquímicas (Udenigwe *et al*., 2021). En este estudio, tras la digestión gastrointestinal de las leches, los péptidos más pequeños (< 0.29 kD, 0.29-0.62) se derivaron de la leche sin fermentar y de FM-572. Por lo tanto, se sugiere que estos péptidos fueron eficientemente absorbidos. De las características fisicoquímicas, la hidrofobicidad/polaridad dada por la composición de aminoácidos influye de igual forma, de manera positiva o negativa en el grado de absorción. Se ha reportado que, por ejemplo, péptidos hidrofóbicos y anfipáticos pueden

unirse a sales biliares en el lumen intestinal. Lo anterior resulta en decremento de la absorción de péptidos y de las sales biliares (Boachie *et al*., 201). Interesantemente, las fracciones peptídicas de FM-600, después de la digestión gastrointestinal, mostraron capacidad de unión a sales biliares. Por esta razón, se podría explicar la baja absorción de los péptidos de FM-600 (Rendón-Rosales *et al*., 2019).

La actividad inhibidora sobre HMG-CoA reductasa se evaluó en las fracciones peptídicas derivadas de las leches fermentadas antes y después de ser sometidas a un proceso de digestión gastrointestinal y de absorción intestinal *ex vivo*. Los resultados mostraron que las fracciones peptídicas ejercieron capacidad para inhibir la enzima HMG-CoA reductasa. Específicamente las fracciones derivadas de FM-600 presentaron mejor eficiencia de inhibición. Centrándonos en el modelo de absorción *ex vivo,* a pesar de que los péptidos de FM-600 fueron menos absorbidos, sorprendentemente fueron los que ejercieron mejor actividad inhibitoria sobre esta enzima ($p<0.05$) comparado con FM-572 y la leche no fermentada. Posteriormente, la fracción absorbida de FM-600 se fraccionó mediante RP-HPLC. Los péptidos que eluyeron primero (Fracción 1) mostraron la mejor eficiencia de inhibición. Lo anterior sugiere que dentro de la fracción 1, se encuentran presentes los péptidos más activos y que presentan características hidrofílicas. En un estudio realizado por Pak *et at*. (2006) se encontró que la asociación entre la actividad inhibitoria de los péptidos sobre HMG-CoA reductasa, no dependía de la hidrofobicidad de la secuencia. Sus experimentos sugirieron que la conformación y los grupos funcionales laterales juegan un papel más importante para inhibir dicha enzima. En reportes previos, indicaron que la presencia de grupos aromáticos, principalmente de fenilalanina en el extremo carboxilo terminal (Silva *et al*., 2021). Además, la presencia de ácido glutámico en el extremo C-terminal también parece jugar un papel en el reconocimiento del sitio activo de la enzima (Pak *et al.*, 2006). Sin embargo, es necesario análisis adicionales para establecer la estructura-actividad de los péptidos absorbidos.

Para confirmar el efecto hipolipidémico de las fracciones de bajo peso molecular (FSA< 10kDa) de las leches fermentadas FM-572 (FSA-572) y FM-600 (FSA-600), las fracciones fueron administradas a ratas hipercolesterolémicas durante cinco semanas. Al final del periodo experimental, los grupos tratados con estas fracciones mostraron menor ganancia de peso corporal en comparación con el control hipercolesterolémico ($p<0.05$). La administración de estas

fracciones redujo los niveles de colesterol total en un 21.21 a 24.3% y C-LDL/VLDL en un 29.7 a 30.2% ($p<0.05$). La reducción de estos lípidos condujo al decremento del riesgo cardiovascular (índice aterogénico en plasma y relación no-HDL/C-HDL). No obstante, el efecto fue significativamente mayor con la administración de FSA-572. Además, esta fracción también redujo los niveles de triglicéridos y ácidos grasos no esterificados (NEFA) en plasma, como consiguiente se observó una mayor excreción de NEFA en heces. Interesantemente, a diferencia de la administración de las leches fermentadas, las fracciones peptídicas (FSA-572) aumentaron la excreción de colesterol en heces. Lo anterior podría indicar una regulación de la absorción de colesterol por diferentes mecanismos, como lo es la inhibición de las micelas de colesterol y posiblemente un efecto menor sobre la microbiota intestinal. Un efecto menor sobre la microbiota podría sugerir una menor abundancia de *Eubacterium coprostanoligenes* y por lo tanto, una mayor excreción de colesterol.

El estudio se complementó con la evaluación e identificación de péptidos potencialmente hipocolesterolémicos derivados de FSA-572 a través de la inhibición micelar del colesterol. Para ello, la fracción (FSA-572) se fraccionó mediante RP-HPLC. Las fracciones colectadas (15 fracciones) mostraron capacidad para inhibir la formación de micelas de colesterol. No obstante, la fracción 3 (F3) fue la que presentó la mayor eficiencia de inhibición. Ocho péptidos fueron identificados en la fracción más activa (F3), los cuales, de acuerdo con sus propiedades fisicoquímicas, presentaron una hidrofobicidad media-alta (escala Wimley-White), ya que la composición de aminoácidos hidrofóbicos fue mayor. Específicamente las secuencias QEPVLGPVRGPFPIIV, DMPIQAFL y ADGVAFV presentaron aminoácidos hidrofóbicos por arriba del 60%. De acuerdo con reportes previos, una alta hidrofobicidad es la propiedad que mejor explica el efecto hipocolesterolémico de los péptidos, especialmente aquellos que inhiben la formación de micelas de colesterol (Zhang *et al*., 2012). Además, se ha reportado que péptidos con dos residuos de aminoácidos ácidos y dos aromáticos en la secuencia tienen influencia en la disrupción de las micelas. En este estudio el péptido NICNISCDKFLDDDLT presentó esta característica.

Un análisis adicional de digestión gastrointestinal *in silico* mostró que los péptidos liberados mostraban potencial efecto hipocolesterolémico por otros mecanismos. Por ejemplo, los péptidos

GPF, GVF y ENF presentaron fenilalanina en su extremo C-terminal. Se ha descrito que la presencia de este aminoácido favorece la inhibición de la enzima HMG-CoA reductasa, inhibiendo, por lo tanto, la síntesis de colesterol (Silva *et al*., 2021). De hecho, los péptidos GPF y GVF exhibieron una alta probabilidad de ser bioactivos de acuerdo con la herramienta Peptide Ranker (puntaje>0.8). Cabe mencionar que los péptidos identificados después de la digestión *in silico* han sido previamente reportados con otras actividades biológicas. Estas actividades incluyen la actividad antioxidante, inhibidores de Dipeptidil-peptidasa IV (DPP-IV) e inhibidores de la enzima convertidora de angiotensina (ECA). Es importante mencionar que el péptido QEPVLGPVRGPFPIIV identificado en la fracción activa mostró propiedades multifuncionales asociadas a la salud cardiometabólica (ver manuscrito 6). Los hidrolizados de QEPVLGPVRGPFPIIV tras el proceso gastrointestinal *in vitro* mostraron actividad inhibidora de trombina, DPP-IV y ECA. Por lo tanto, además de estas propiedades, se puede sumar al potencial efecto hipocolesterolémico. No obstante, se requieren estudios adicionales *in vivo e in vitro* para establecer el efecto hipocolesterolémico de los péptidos identificados en la fracción más activa.

En resumen, los hallazgos de la presente tesis indican que las leches fermentadas estudiadas, específicamente las leches fermentadas con NRRL B-50572 y NRRL B-600 muestran capacidad para reducir los niveles de colesterol, lo cual puede asociarse a la mejora de la funcionalidad de la microbiota intestinal, aumentando la proporción de bacterias benéficas relacionadas a la producción de butirato. Por otra parte, las fracciones peptídicas derivadas de las leches fermentadas mostraron potencial efecto hipocolesterolémico. Los potenciales mecanismos hasta el momento estudiados podrían involucrar el incremento de la excreción de colesterol asociado a la inhibición de la solubilidad micelar del colesterol, y la posible inhibición de la enzima HMG-CoA reductasa. Se requieren estudios adicionales para estudiar los mecanismos subyacentes, así como el efecto benéfico de estas leches fermentadas en un estudio clínico.

8. CONCLUSIONES

El consumo de leches fermentadas con cepas de *L. lactis* NRRL B-50572 y NRRL B-50600 ejerce efecto hipocolesterolémico en ratas Sprague-Dawley hipercolesterolémicas, al disminuir los niveles de colesterol total y colesterol LDL.

El consumo de leches fermentadas con cepas de *L. lactis* NRRL B-50572 y NRRL B-50600 previene la acumulación de lípidos hepáticos (colesterol y triglicéridos) en ratas hipercolesterolémicas.

La microbiota intestinal mostró una respuesta positiva con el consumo de leches fermentadas, al mejorar grupos específicos bacterianos a nivel colónico y fecal, y mejorar los niveles de ácidos grasos de cadena corta.

Los grupos bacterianos, *Eubacterium coprostanoligenes*, Lactobacillaceae y Ruminococcaceae se asociaron negativamente con los niveles de colesterol LDL y positivamente con butirato. Además, el butirato se asoció negativamente con los niveles de colesterol total y LDL, y positivamente con HDL.

Las leches fermentadas con *L. lactis* NRRL B-50572 y NRRL B-50600 liberan fracciones peptídicas potencialmente bioaccesibles con capacidad para inhibir la formación de micelas de colesterol, e inhibir a la enzima HMG-CoA reductasa.

La fracción de bajo peso molecular (F < 10kDa) proveniente de la leche fermentada con *L. lactis* NRRL B-50572 ejerce efecto hipolipidémico mayor que con *L. lactis* NRRL B-50600, al disminuir significativamente los niveles de colesterol C-LDL, triglicéridos, ácidos grasos libres y mejorar los niveles de C-HDL, y aumentar la excreción de colesterol.

La caracterización parcial *in vitro* e *in silico* de la fracción peptídica indicó que, la leche fermentada con *L. lactis* NRRL B-50572 libera péptidos con capacidad para inhibir la formación de micelas de

colesterol.

El consumo de LF-572 pudiera ser utilizada como coadyuvante en el manejo de la dislipidemia, principalmente para reducir los niveles de colesterol.

9. RECOMENDACIONES

El presente estudio evaluo el efecto en la respuesta de los niveles de lípidos por el consumo de leches fermentadas por cepas de *L. lactis*, lo cual podría estar asociado a la participación de péptidos bioactivos en conjunto con la microbiota intestinal en la regulación de los niveles de lípidos. Se sugiere, primero en profundizar en la identificiación de péptidos y comprobar cuales de las secuencias son responsables del efecto hipocolesterolémico mediante estudios in *vitro* e *in vivo*. Posteriormente se sugiere de los estudios de los mecanismos moleculares involucrados en dicho efecto mediante estudios de expresión génica y de proteinas del metabolismo del colesterol. También se sugiere la evaluación del efecto hipocolesterolémico de la leche fermentada en co-cultivo (*L. lactis* NRRL B-50572 y NRRL B-600). Finalmente, se requieren estudios de diseño de la leche fermentada hipocolesterolémia para consumo y evaluar el efecto en estudios clínicos.

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